Journal of
DAIRY SCIENCE

Vol. 42 July, 1959 Number 7

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Symposium on Forage Evaluation

A combined reprint of the eight papers on Forage Evaluation, given at the Intersociety Symposium at Purdue University, August, 1958, and appearing in the Agronomy Journal, Vol. 51, April, 1959, are available. These reprints, at 80 cents each, may be ordered from the American Society of Agronomy, Central Office, 2702 Monroe Street, Madison 5, Wisconsin.

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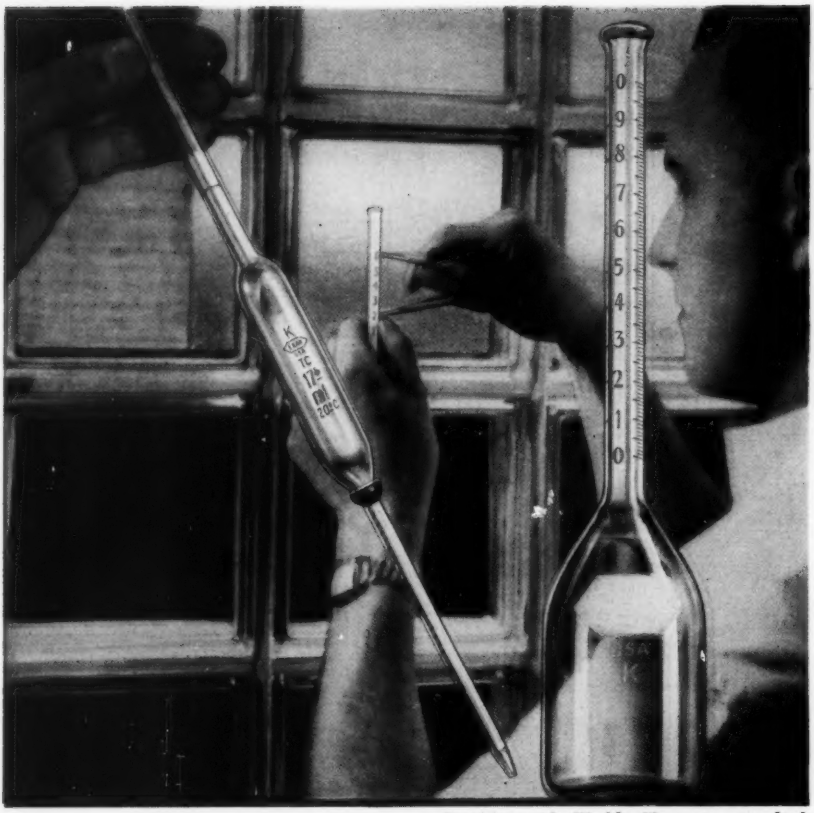

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PEOPLE AND EVENTS

COLLEGE AND UNIVERSITY NEWS

Illinois

K. E. GARDNER, Professor of Dairy Science at the University of Illinois, will become Associate Dean of the College of Agriculture at that school on August 1, 1959.

Dr. Gardner has been a member of the College of Agriculture staff at the University of Illinois since 1940, when he was named an extension specialist of dairy husbandry. His first work was to train and supervise DHIA testers and to help conduct feeding schools.

K. E. Gardner

He was raised on a dairy farm near Indianapolis, Ind., and was a member of one of the early 4-H clubs in that part of the state. He has shown dairy cattle and swine at many local and state fairs. He attended Purdue University from 1932-1936, majoring in the field of dairy husbandry. He worked as a graduate assistant in teaching and research at Cornell University from 1936-1940 and received both M.S. and Ph.D. degrees there.

From 1943 to 1946, he served as captain in the Sanitary Corps of the Medical Department in the United States Army. He saw two years of service in Europe with the 3rd U. S. Army. He was responsible there for the ration and nutrition of prisoners of war and displaced persons, as well as for hospital patients from the 3rd Army.

He rejoined the University of Illinois staff in 1946. He has served as chairman and member of the University Committee on Student Affairs for four years, and chairman of the Students-Grants-In-Aid Committee for three years. He has also been chairman of the Course and Curriculum Committee, the Enrollment and Student Relations Committee and Extra Mural Courses Committee in the College of Agriculture. He has also been a member of the Executive Committee of the College of Agriculture for four years, and adviser to Alpha Gamma Rho, social fraternity for 17 years, member of the Champaign County Red Cross Board, and president of the Village Board of Tolono, Ill. He is a major in the U. S. Army Reserve.

From April to July, 1957, and again in April, 1958, Dr. Gardner was in Turkey as deputy director of a Military Survey Team appointed to investigate nutrititional deficiencies in personnel of the Turkish Military Services. This project was a joint effort of the United States and Turkish governments.

As Associate Dean of the College of Agriculture, Dr. Gardner will be in charge of all resident instruction in Agriculture and Home Economics. His duties will include such items as courses and curricula, student counselling, scholarship, and student activities.

California

G. J. SMITH, on leave from Australia, and attending University of California at Davis as a graduate student in food science, was awarded the William V. Cruess Scholarship for 1959. This scholarship, which is admintered by the Northern California Section of the Institute of Food Technologists, consists of $200, an award certificate, and a one year student membership in both the National Institute of Food Technologists and the Northern California Section of the Institute.

The award is made annually to a graduate student in the field of food science, microbiology, biochemistry or any related natural science who is carrying at least ten units, and is pursuing a career in food science.

Louisiana

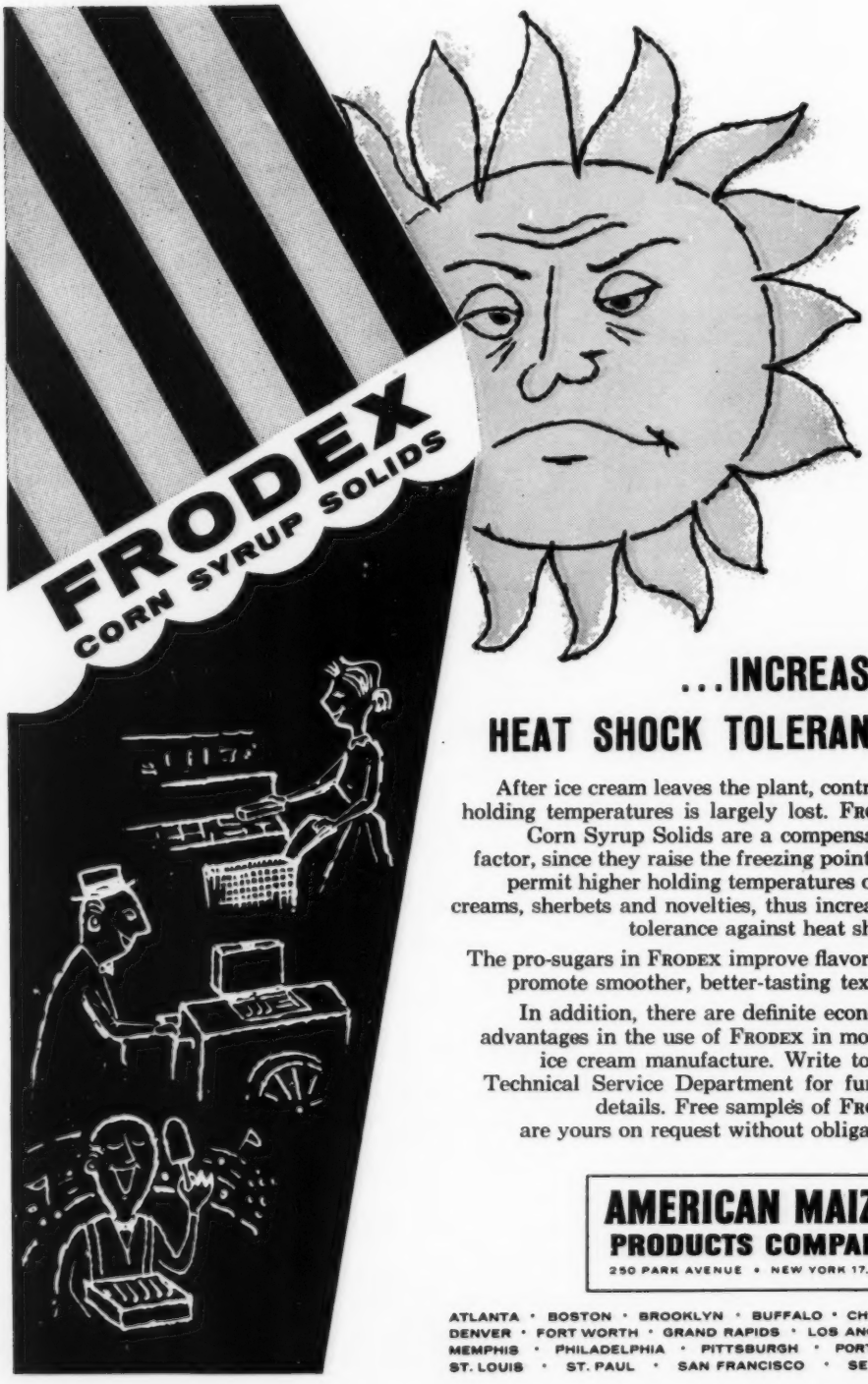
The Louisiana annual heifer sales of Guernseys, Holstein-Friesians, and Jerseys were held during April and May.

At the Guernsey sale held at Walker's Cloverland Dairy, Alexandria, 25 open heifers, selected in Indiana through the Indiana Guernsey Cattle Club, sold for an average of $212. The highest price paid was $345 by JOHN GREEN, JR. of Minden, Louisiana. These animals, mostly under a year of age, were especially suitable for Junior Club Member projects.

The Holstein sale at Shreveport averaged $427 for 41 heifers, most of them bred. Thirtynine of these animals were consigned by the Fox River Valley Holstein Breeders' Association, Fond Du Lac, Wisconsin. The highest price paid was $550 by L. E. GATES of Greenwood, Louisiana, for a bred heifer.

The Jersey sale held at the Dean Lee Agricultural Center near Alexandria, contained 34 heifers, mostly open, consigned by 21 Louisiana breeders and one Arkansas breeder. The average price was $171; the highest price, $325, for a heifer bred by the Louisiana State University was paid by SULLIVAN AGUILLARD of Oberlin, Louisiana.

The trend in these spring sales is toward more interest in bred heifers, ready to take a place in a dairy herd by fall. Calves suitable for club projects are becoming more available throughout the state since the widespread use of artificial insemination and can often be



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Nebraska

Twenty-eight persons received certificates signifying successful completion of a 12-week Dairy Plant Management short course sponsored by the University of Nebraska Extension Division and Dept. of Dairy Husbandry.

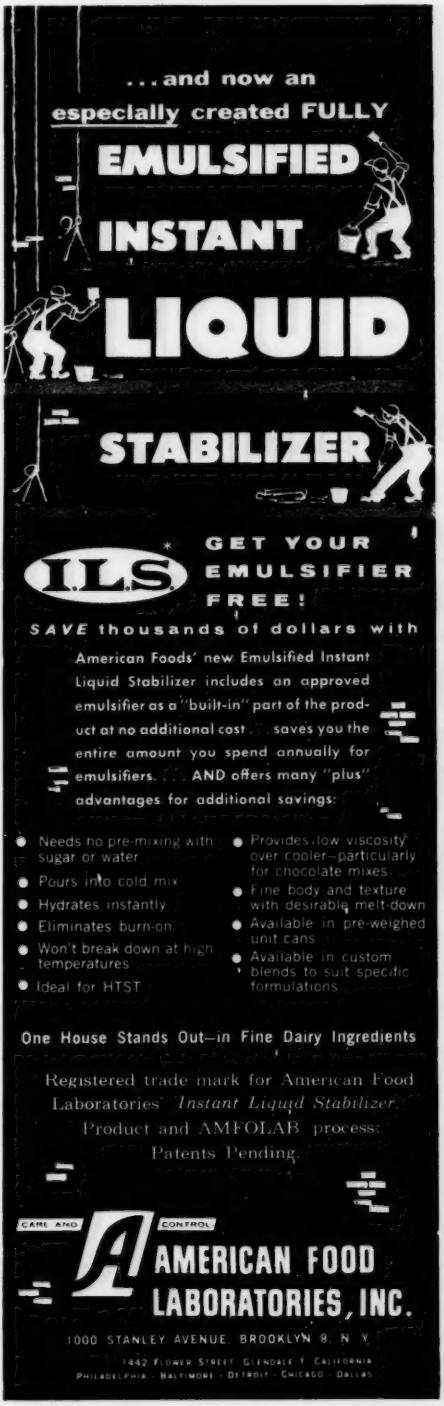
Those enrolled in the course included primarily milk and ice cream plant personnel and the Univecsity Dairy Department. The from four dairies in Lincoln, one in Seward course is designed to train the plant employees in basic dairy technology, management and plant work.

The certificates were presented by W. V. LAMBERT, Dean of the University's College of Agriculture. NILES BARNARD, Chairman of the University Mechanical Engineering Department, spoke on "Foremanship," and KEN KELLER, assistant director of University Public Relations, spoke on public relations.

Back row (1 to r)—P. A. Downs, E. Zlab, B. O. Worley, A. Lickei, J. H. Bowman, D. L. Fletchall, V. S. Irons, M. Monson, B. Schirmer, D. D. Ehrlich. Second row (standing)—W. V. Lambert, M. Lang, W. F. Schnell, W. D. Weaver, L. Meyer, G. Lebsack, K. A. Hays, R. E. Austin, G. Hilblink, J. D. Sturn, C. A. Meyers, G. O. Knight, R. Scharton, Khem Shahani, D. L. Crosby, M. Nilson, T. A. Evans. Front row—P. L. Kelly, N. E. Bean, L. F. Bryant, R. E. Null, J. C. Hagadorn, F. M. Rulla, E. L. Sterns, Dr. R. B. Maxcy.

New York

About 150 milk processors, dairy plant fieldmen, and milk regulatory officials gathered from New York and neighboring states to discuss the precautions that need to be taken to produce and maintain milk of the highest quality. The first day of the conference was devoted to a milk judging clinic and a discussion by the Dairy Industry staff of the causes of off-flavors in milk. A dinner was held on May 5 at which time HERRELL DEGRAFF, Professor of Food Economics at Cornell University, spoke on "The Competitive Position of North Eastern Fluid Milk." The second day was devoted to a discussion of UHT and flavor removal equipment. Guest speakers included E. O. HERREID, University of Illinois; A. C. SMITH, University of Connecticut, and JOHN BARBER, Taylor Instrument Companies.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(1) Standard Methods for the Examination of Dairy Products, ed. 10, New York, American Public Health Association, 1953. (2) Committee Report, Am. J. Pub. Health 42:1131 (Sept.) 1952. (3) Microbiological Methods, report at 66th Ann. Meet. Assn. Official Agricultural Chemists, Sept. 29, 1952: J. Assn. Official Agr. Chem. 36:91 (Feb.) 1953. (4) Standard Methods for Examination of Water, Sewage and Industrial Wastes, ed. 10, New York, American Public Health Association, 1955.

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North Carolina

The Dept. of Animal Industry held its first Livestock and Dairy Industries Career Day in May. One hundred and twelve high school boys representing 63 high schools were brought to the campus by 60 sponsors. Tours were made of the facilities for training the students, demonstrations of types of research that are underway were given and activities of the department and job opportunities were pointed out by faculty, students and alumni. The guests were entertained with a barbecue luncheon, livestock and dairy show, and a movie "This Is the Dairy Industry" as well as highlights of the athletic program at N. C. State College.

J. E. LEGATES recently returned from a visit with R. K. WAUGH in Bogota, Colombia, S. A., where he reviewed the breeding projects underway which are sponsored by the Rockefeller Foundation. The opportunities for growth of the dairy and livestock industries in that country were most impressive.

R. B. FARTHING, Dept. of Animal Industry, has accepted a position of Experiment Station Statistician and Professor of Dairy Breeding at Louisiana State University, Baton Rouge, Louisiana. He will assume his new duties July 1, 1959.

Oregon

Related symposiums on food enzymes and molecular biology have been scheduled for September at Oregon State College with food technologists and biochemists from throughout the West expected to attend.

The "Symposium on Foods: Enzymes" will be held September 9-10 with the second symposium on "Perspectives in Molecular Biology" planned September 11-12.

The food technology department is sponsoring the symposium on enzymes as the first in what is expected to become a continuing series of conferences on important food industry topics. HAROLD SCHULTZ, head of the Dairy and Food Technology Dept. at the college, is symposium chairman.

Purpose of the symposium is to emphasize the important role of enzymes in food processing, he noted, and new research developments on enzyme properties and actions.

Opening highlight of the symposium will be a special program honoring ERNEST WIEGAND, who founded the nation's first food technology department at Oregon State in 1919. He retired from the college staff in 1953 but is still active as a food industry consultant.

The symposium on molecular biology will be the annual meeting of the Pacific Slope Biochemical Conference. Some 300 scientists from eight western states and British Columbia are expected to take part in the discussions.

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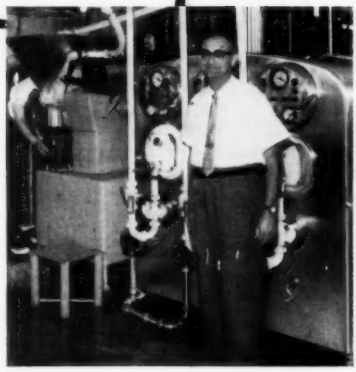

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This symposium will highlight the important major trends in fundamental chemical biology. It is planned in part to commemorate the completion of new research laboratories at Oregon State College, which will be devoted to basic research in the life sciences.

VERNON CHELDELIN, director of the OCS Science Research Institute, will be symposium chairman.

Housing in campus dormitories is being arranged for scientists attending the two conferences. Both conferences were scheduled as a part of the Oregon Centennial Year series of scientific meetings sponsored by Oregon State College.

Pennsylvania

The Pennsylvania State University Dairy Science Club held its annual election of officers with the following results: president, CHARLES ITLE; vice president, MELVIN DURBORAW; secretary, MARILYN NEWLIN; treasurer, WESLEY TRUMBAUER.

Virginia

Twelve seniors received B.S. degrees in June. Five were dairy technology majors and seven were dairy production majors. Included in the group was JUDITH HESS, graduating with honors. She had the highest quality point average of all V.P.I. women seniors. She also received the Borden Scholarship Award and the A.A.W.U. Scholastic Award. ROBERT BENTON was president of his class and the civilian student body and chancellor of Alpha Zeta. Both of these graduates, along with JOHN GARBER and WILLIAM HARRIS were listed in the latest edition of Who's Who Among Students in American Colleges and Universities.

P. M. REAVES attended the Education Workshop held at the University of Tennessee in June. He was one of three members of the resident teaching faculty in the School of Agriculture to attend this workshop.

A bulletin containing data collected from a survey made of the Grade A dairy farms in Virginia came off the press this month. The information in the bulletin will be presented mostly in chart and tabulation form and is very comprehensive in nature. This is the first of a series of bulletins that are expected to be published which will contain information on all segments of the Virginia dairy industry. A survey of the dairy plants is now nearing completion and a survey of milk producers is

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now in the planning stage. The surveys are a cooperative project of the Virginia Dept. of Agriculture and the Virginia Agriculture Extension Service.

West Virginia

The Dairy Science Club at West Virginia University has been very active during the past school year. The members are looking on a successful year with satisfaction and are looking forward to an even greater year ahead under president-elect DON BUTCHER of Mineral Wells. Don has been a hard working member during the past two years. He works at the University Dairy Farm, won grand champion showman honors at the spring Dairy Cattle Congress (a Dairy Science Club activity), and was named the Club's delegate to this spring's all campus Leadership Conference. He will be a senior next year and plans to be married this summer.

The incoming vice president, HAYMOND LUZADDER, Grafton, also works at the Dairy Farm. A senior next year, he was champion Holstein showman at our Cattle Congress. The secretary will be CARROLL ROGERS, Spencer, a dairy technology major who does part-time work in the University Milk Plant. JOHN BARGELOH, Mineral Wells, will take care of the Club's finances.

Under coach RALPH MITCHELL, the dairy cattle judging team competed in the contest at Eastern States Exposition. The dairy products team, coached by SAM WEESE, took part in the International Contest at Chicago.

The Dairy Club's basketball team, under the versatile coach Mitchell, again took part in the University intramural program and were successful in winning more than half of the games played.

The student Dairy Cattle Congress was well attended and, as usual, a real success. Reigning over the affair was RAMONA MATLICK of Fellowsville, who was crowned by the Director of the Experiment Station A. H. VAN LANDINGHAM. KENNETH BAILEY, McComas, a senior in dairy technology, served as Master of Ceremonies.

Completed Theses

M.S. Degree

Mrs. MARY KNIGHT KIMZEY—The effect of heating cream in a vacreator on some of its properties. Virginia Polytechnic Institute, Blacksburg.

VIGGO BRANDSTRUP ANDERSEN—Relation of culture composition to the flavor of cultured buttermilk and sour cream. University of California, Davis.

JOSEPH LEE III—Protein-bound iodine in the blood of dairy cattle and its relation to growth and milk production. Clemson College, Clemson, S. C.

GOVERNMENT AGENCY NEWS

Taft Center Course Announced

The Robert A. Taft Sanitary Engineering Center, Cincinnati, announces a two-week training course, "Radionuclides in Foods," for September 14-25. Designed for professional personnel responsible for the surveillance of radioactive materials in milk and foods, the course will provide training in methods of sampling and assay of radioactive contaminants and in procedures for data interpretation, and will offer opportunity for discussion of problems in this environmental area.

Introductory sessions will be devoted to radiation fundamentals and instrumentation: (1) sources of radionuclides in food, (2) aquatic food chains, (3) terrestrial food chains, (4) milk and dairy products, (5) sampling procedures, (6) radiochemical procedures, (7) maximum permissible concentrations, and (8) public health significance of radionuclides in foods. Later sessions will be devoted primarily to laboratory demonstrations. Specialized facilities and equipment required by the course limit the number of trainees who can be accommodated.

Applications or requests for additional information should be addressed to the Chief, Training Program, Robert A. Taft Sanitary Engineering Center, 4676 Columbia Parkway, Cincinnati 26, Ohio, or to a PHS Regional Office Director.

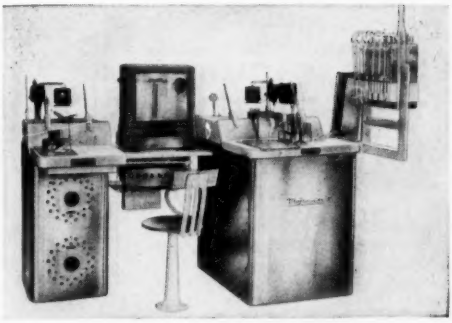
Gordon Receives USDA Award

WILLIAM GORDON was honored recently in Washington as a recipient of the USDA's Superior Service Award. A plaque was presented to Dr. Gordon for his outstanding research in the chemistry of milk proteins by Secretary of Agriculture EZRA TAFT BENSON. The ceremony was held in the Sylvan Theater, on the grounds of the Washington Monument.

Wm. Gordon

Since 1941, Dr. Gordon has been on the staff of the Agricultural Research Service's Eastern Utilization Research and Development Division. His research on milk proteins has been important in establishing the nutritional properties of milk. He perfected a method for isolating the unique milk protein alpha-lactalbumin, and studies of this pure protein have established its unusual nutritional significance.

Dr. Gordon's research has also provided a complete analysis of the amino acids in the milk proteins alpha-casein, beta-casein, gamma-



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casein, unfractionated casein, and beta-lactalbumin. Essentially all of the amino acids in the proteins of milk have been accounted for by this work, both qualitatively and quantitatively. Last year Dr. Gordon's research earned for him the $1,000 Borden Award in the Chemistry of Milk.

Dr. Gordon is a native of New York City, and is a graduate of Cornell University. He received his Ph.D. degree from Yale University, where he served successively as teaching assistant, National Research Council fellow in medicine (physiological chemistry), and Coxe fellow in physiological chemistry. He was an instructor in biochemistry at Stanford University, and undertook research on milk proteins as a Smith, Kline, and French fellow at Pennsylvania State University before he joined the U. S. Department of Agriculture.

Dr. Gordon is a member of the American Society of Biological Chemists, American Chemical Society, American Association for the Advancement of Science, New York Academy of Science, Sigma Xi, and Phi Lambda Upsilon.

INDUSTRY NEWS

M.I.F. NEWS

Two British dairy officials whose company delivers milk to the royal family of England, were among the 16 dairy officials who were graduated from the 70th session of the Sales Training Institute of the Milk Industry Foundation.

DAN LANGLEY and G. W. SMITH, Express Dairy Co., Ltd., London, attended the two-week basic course of study with sales officials of milk companies representing every section of the continental U. S. Their dairy is under commission to serve the Queen's Household and other royalty.

This brings the total number of graduates to 1,263 since the Institute began eight years ago. The Institute is operated as a sales-building service to MIF members.

A. E. Staley News

K. N. WRIGHT has been named director of nutrition in the A. E. Staley Manufacturing Co.'s formula feed department. Wright succeeds W. N. MCMILLEN, who resigned to develop a private hog breeding enterprise.

Wright has been associated with animal nutrition work for 18 years. He earned his bachelor's and master's degrees at the University of Illinois, where he was a staff member in animal husbandry and nutrition before joining Staley's in 1946.

He was U. S. representative as poultry nutrition specialist at the Palermo, Italy, 1957 International Agricultural Fair, sponsored by the Soybean Council of America, the Foreign Agricultural Service, and the USDA.

In addition to his extensive research work, he has worked with feed manufacturers over

the country on nutrition and consultation as Staley feed nutritionist since 1953.

Dr. McMillen, as director of nutrition since 1951, has been one of the chief architects of Staley's feeding programs and feed lines. He will continue his association with Staley's on an independent consulting basis.

Klenzade News

MYRON HALES has been appointed manager of the new Cultured Products Division of Klenzade Products, Inc., Beloit, Wis. This new department has been organized to offer specialized technical services to manufacturers of cheese and various cultured products such as cheddar and cottage cheese, buttermilk, sour cream, etc.

Myron Hales

Mr. Hales is a graduate of the University of Wisconsin in dairying and holds a master's degree from the University of Missouri. He was formerly manager of Chr. Hansen's Laboratories, Inc., Milwaukee, Wis., and has devoted many years to the development, handling, and application of cultures to cultured dairy products and is the author of numerous bulletins and articles on the subject. He has addressed scores of meetings and conventions and is in wide demand as a technical speaker with an international reputation on cultured dairy products manufacturing problems.

The addition of Mr. Hales to the Klenzade staff and the creation of this new division marks another forward step in the Klenzade program of constantly expanding services to the dairy and food industries.

Borden's News

LYNN CHRISTIAN has been promoted to technical advisor of the Borden Special Products Company's feed supplements department.

Mr. Christian served as field service nutritionist at Borden's Nutritional Research Laboratory in Elgin, Ill., before joining the feed supplements department's home office in New York City four years ago.

The new technical advisor received his bachelor's and master's degrees in animal husbandry from Pennsylvania State University. A veteran of the U. S. Army, he saw active duty during both World War II and the Korean conflict.

D. RICHARD KNAUFF has been named a research and development coordinator of the Borden Special Products Company.

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CHR. HANSEN'S LABORATORY, INC.
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Dr. Knauff will be located at the company's Product Development Laboratory in Elgin, Ill. He will be in charge of coordinating research and development activities in animal feed supplements.

Prior to joining Borden's, Dr. Knauff was associated for two and a half years with Hess and Clark, Inc., Ashland, Ohio, a feed medication firm. Earlier, he was on the faculty of the University of Minnesota, where he earned his master's degree. He received his doctor's degree in veterinary medicine from the University of Georgia and his bachelor's degree in dairy technology from the University of Maryland.

During World War II, Dr. Knauff served two years with the U. S. Marines' war dog detachment, training canines for combat duty.

INTERNATIONAL NEWS

D.S.I. in Spain

Something new in U. S. participation in International Trade Fairs found the Dairy Society International's complete, working dairy plant the center of attention in Madrid, Spain, in May and June. The entire U. S. display was built around the dairy demonstration where recombined milk and ice cream from nonfat dry milk and anhydrous milk fat were sampled. Supervising the display (the Society's third in Spain) are GORDON LAMONT, Darien, Conn., Nestlé director and chief-of-mission; P. E. ELLIKER, Oregon State College, Corvallis, and J. BAZO, Cincinnati, Ohio. Dry Milk Products will be furnished by the USDA's Foreign Agricultural Service, which is in charge of the U. S. participation program.

The dairy demonstration was chosen to highlight the Fair because Madrid is an area where there is real need for a market milk supply. Pasteurizing is limited and milk production in the area does not meet the city's potential milk requirements. Recombining pure milk fat and nonfat dry milk has created a great interest among Spain's dairy officials who are seeking means of supplementing the local supply with a quality product.

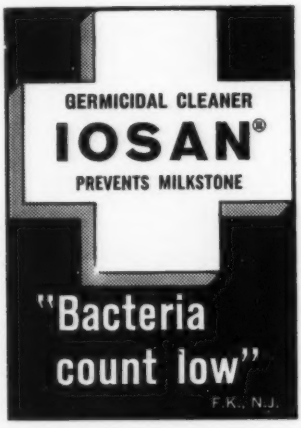
Chief-of-mission Lamont preceded his other two team members by several weeks to lay the groundwork for a successful showing, and to interest dairy leaders in recombining. Lamont has done spadework for many of the Society's other projects. It was he who did much of the preliminary planning for the two long-term educational projects in Colombia and Thailand. This is his first DSI mission to the European area.

D.S.I. in India

Dairy Society International is now in the preparatory stage for the third of a series of demonstrations of recombining milk and making ice cream in India in connection with Small Industries Fairs sponsored by the U. S. Department of Commerce. First of these, in December, 1958, was held in New Delhi, the capital, where the DSI team, L. C. KENWORTHY, Meadow Gold Dairies of California, Pasadena; GEORGE MCCONNELL, Foremost Dairies, Inc., San Francisco; K. M. SHAHANI, Associate Professor, Dept. of Dairy Husbandry, University of Nebraska, Lincoln, had the opportunity to serve Prime Minister NEHRU, among many others, samples of milk and ice cream turned out in the full-scale demonstration plant. Equipment, as in other DSI Fairs demonstrations, was loaned through the Society of Manufacturers, and the Foreign Agricultural Service assisted by providing milk ingredients.

This was followed by a similar demonstration in Calcutta—March and April—with the DSI team this time consisting of J. S. DETWILER of the Taylor Instrument Companies, and G. H. WILSTER, recently retired from the Dairy and Food Technology Dept. at Oregon State College. In Calcutta, sampling was for special industrial, governmental and educational groups only, but product was distributed to a number of charitable institutions and schools. The third in the series is Madras, which is scheduled for mid-August.

Going directly from his DSI duties at the Calcutta Fair, Dr. G. H. Wilster started an assignment with FAO as technical advisor to the Kaira District Milk Producers, Ltd. at Anand, India. Dr. Wilster, who has published numerous scientific papers in journals in all



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parts of the world, will remain at Anand for approximately eight months helping in the development of evaporating operations in this area which furnishes a substantial amount of the milk for Bombay's pioneering Milk System.

Veterinary Services In Israel

The establishment of the State of Israel in 1948 saw great strides in the development of animal husbandry. The number of cattle during the past ten years has increased by over 100%, with an average annual milk yield of 4,300 litres, which compares well with countries such as Holland.

Sheep are raised both for milk and meat purposes, and their number has increased from 29,000 to 135,000 in 1958.

In 1948, poultry breeders failed to supply the nation's needs. Today, although the population has more than tripled, Israel is able to export 40 million eggs a year, a rise in some 250%. During this period, the raising of beef cattle was initiated, and large herds of beef cattle are maintained on the range. The animal husbandry branch appears to be well on its way to achieve the ultimate purpose of supplying the nation's needs in animal products.

The safeguarding of the animal industry against diseases and the efficient control of diseases enzootic to our country is the primary duty of the Veterinary Service, and this is made possible largely through the work of the Veterinary Institute at Beit Dagan. The Veterinary Institutes activities may be classified in three major fields: diagnosis, research, and vaccine production.

A staff of more than one hundred is engaged at the Institute, whose origins range from China to Switzerland, from Yugoslavia to the United States.

The research projects at the Institute reflect the needs of Israel's animal husbandry branch. The most important research projects in which the Institute is engaged at present involve rabies, foot & mouth disease, leptospirosis, agapactia, brucellosis in sheep, enterotoxemia, gangrenous mastitis in sheep, adenomatosis, and tick fevers.

Production of vaccines is another task of the Veterinary Institute. Most of the animal industry's requirements in vaccines are produced and supplied by the Veterinary Institute. Some of the vaccines produced, such as newcastle, modified avianized rabies vaccine, fowl pox for day-old chicks, have been developed at the Institute's own laboratories. It is hoped that in the near future, foot & mouth disease vaccine will also be produced there.

DAIRY TECHNOLOGY SOCIETIES

Atlanta—A social event consisting of dinner and a floor show at an Atlanta hotel served as the June meeting of the Atlanta society. The event was a night out "without speches" for a year's work well done.

Detroit—An annual father and son-daughter banquet was held by Detroiters. Prof. P. S. LUCAS was the featured attraction with films from his personal file.

Kansas City Area—"The Responsibility of Supervisory Help in Labor Relations in the Dairy Industry," was the main speaker's topic at the May 28 meeting. Speaker was C. R. HOSTER, business agent, Milk Drivers and Dairy Employees Union, Local 207, Kansas City, Mo.

Michiana—An annual outing of golf, a chicken dinner, swimming, and the like highlighted the June meeting. This was the 15th annual outing for the society.

Tri-State—New officers for the 1959-60 season are P. F. DEMBLOWSKI, All Pak Inc., Pittsburgh, president; ROY GINN, Sealtest, Rieck Div., Pittsburgh, vice president; DAVID MIZER, Hillcrest Dairy, Cadiz, O., second vice president; LOU FRESA, Meadow Gold Dairies, Pittsburgh, secretary-treasurer, and W. E. WILKINSON, Dairy Pac representative, Murraysville, Pa., corresponding secretary. Speaker at the May meeting was C. W. BLACKSTOCK, sales representative, Sealright Corp., on the topic, "What Is a Package to You?"

Utah—The May meeting featured PAUL NICHOLS, Dept. of Bacteriology, University of Utah. Dr. Nichols spoke on the topic, "Bacteriological Problems in Liquid Sugar Blends."

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ROLE OF XANTHINE OXIDASE IN THE DEVELOPMENT OF SPONTANEOUSLY OXIDIZED FLAVOR IN MILK [1]

L. W. AURAND AND A. E. WOODS
Department of Animal Industry, North Carolina State College, Raleigh

SUMMARY

The role of xanthine oxidase in the development of spontaneously oxidized flavor was considered. The study included: (1) the relationship of xanthine oxidase activity to the development of off-flavor, (2) a multiple-enzyme system versus a single enzyme in the development of the off-flavor, and (3) the effect of inhibitors upon the development of spontaneously oxidized flavor. Results of the study indicated that: (1) The occurrence of a spontaneously oxidized flavor in milk was dependent upon a high level of xanthine oxidase activity, (2) a single enzyme (xanthine oxidase) was involved, and (3) inhibitors of xanthine oxidase were effective in preventing flavor development. A brief discussion of the peroxidatic action of xanthine oxidase was included.

In 1943, Roberts (19) reported significant correlations between the oxidizing enzymes normally present in milk and the development of oxidized flavor in milk. Dorn (6) stated that a peroxide action upon the milk fat could be traced to the action of xanthine oxidase and ascorbic acid oxidation. He concluded that riboflavin was mainly responsible for the development of oxidized flavor; whereas, ascorbic acid was only secondarily so. Greenbank *et al.* (11) suggested that xanthine oxidase could be of great significance in problems of oxidative deterioration of milk and milk products. Recently, it has been reported (2) that an enzyme is involved in the development of spontaneously oxidized flavor and that the enzyme so involved may be xanthine oxidase. Studies were initiated, therefore, to determine the role of xanthine oxidase in oxidized off-flavor development.

EXPERIMENTAL PROCEDURE

Milk. The milk used during the course of the study was obtained from the college herd. Care was taken to prevent metal contamination and exposure of the milk to the direct rays of the sun. The samples were stored in glass containers at approximately 5° C. and were examined periodically for flavor development.

Enzyme preparations. Xanthine oxidase was isolated from fresh raw cream according to the method of Ball (3). Lactoperoxidase was prepared from fresh whole milk by the method of Elliott (8). A commercial beef liver catalase preparation, Paul-Lewis Laboratories, was used.

Methods. The spectrophotometric method proposed by Dunkley and Jennings (7) was used to measure the development of oxidized flavor. Essentially, it consists of measuring the intensity of color, as optical density, resulting from the reaction between the oxidized flavor components and 2-thiobarbituric acid at a

Received for publication February 13, 1959.

[1] Published with the approval of the Director of Research, North Carolina Agricultural Experiment Station as Paper No. 1002 of the Journal Series.

wave length of 535 mμ. Hereafter, this method will be referred to as the T.B.A. method. The results reported are the means of duplicate determinations and are representative of several trials. As a guide for the interpretation of the data presented, the following T.B.A. scale in optical density readings was used: Slight oxidized flavor, values equal to or greater than 0.055; pronounced oxidized flavor, values greater than 0.070. Xanthine oxidase activity was determined manometrically in air at 37° C., by the method of Ball (3). Activity is expressed in terms of microliters of oxygen absorbed per hour per milliliter of milk, readings during the first 30 min. being employed. Lactoperoxidase activity was measured colorimetrically by the method proposed by Aurand *et al.* (1). Activity is expressed in terms of P.Z. units.[2] Catalase activity was measured by the titrimetric method of Maehley (18). Essentially, this method measures the disappearance of hydrogen peroxide by titration with potassium permanganate. A unit of activity is such that hydrogen peroxide is decomposed at a rate of 12 mg. per minute.

Reagents: (a) folic acid, (b) hypoxanthine, (c) *p*-chloromercuribenzoate (PCMB), (d) 2-amino-4-hydroxy-6-formyl pteridine[3] (6-PA).

RESULTS

General. The results of an earlier study (2) indicated that an enzyme was involved in the development of spontaneous oxidized flavor. It was also suggested that the enzyme involved may be xanthine oxidase. As a result, individual samples of milk were taken from cows in the college herd and were examined both for xanthine oxidase activity and for the flavor defect. Examination of the accumulated data indicated a good correlation between enzyme activity and oxidized flavor development. It was observed that when milks possessed xanthine oxidase activity ranging between 120 and 140 units, flavor development was rapid; whereas, milk possessing an activity less than 100 units failed to develop spontaneous oxidized flavor. Milks possessing an activity between 100 and 120 units were not consistent in the production of an oxidized flavor.

Relationship of xanthine oxidase activity to the development of oxidized flavor. As a result of the observations made in the preliminary study, experiments were designed to ascertain the relative significance of enzyme activity to flavor development. Herd milk was divided into three portions. One portion served as a control, one portion was heated at 63° C. for 30 min., and one portion was heated at 76° C. for 30 min. The xanthine oxidase activity of these portions, after treatment, was 75, 45, and 0 units, respectively. The milks were then subsampled. One portion served as a control, another sample was adjusted to a xanthine oxidase activity level of 138 units, and to another was added a like amount of heat-inactivated xanthine oxidase. All samples were then checked

[2] The activity of peroxidase, P.Z., is expressed in terms of the milligrams of the purpurogallin, i.e., it is the number of milligrams of purpurogallin formed per milligram of enzyme preparation, when it acts upon 5 g. of purpurogallin in 2.1 of water plus 10 ml. of 0.05% hydrogen peroxide for 5 min. at 20° C.

[3] Furnished through the courtesy of Dr. W. W. Williams, Lederle Laboratories Division, American Cyanamid Company.

at zero, one, three, and six days for flavor development by the T.B.A. method. The results of this study are presented in Table 1. It will be seen that flavor development (O.D. > 0.055) occurred only in the samples that were adjusted for enzyme activity. The results obtained for the adjusted heated milk (Sample 8)

TABLE 1

Relationship of xanthine oxidase to the development of oxidized flavor as measured by the thiobarbituric acid method

	Sample No.	Xanthine oxidase added to milk	Xanthine oxidase activity	Days			
				0	1	3	6
			($\mu l.O_2$ uptake/hr./ml)	(O.D.)			
Raw	1	−	75	.018	.020	.030	.028
	2	+	138	.029	.050	.062	.060
	3	+(Inactive)[a]	75	.020	.024	.028	.030
Pasteurized, 63° C. for 30 min.	4	−	45	.017	.020	.025	.028
	5	+	138	.025	.052	.059	.063
	6	+(Inactive)	45	.024	.025	.032	.036
Heated, 76° C. for 30 min.	7	−	0	.024	.025	.030	.027
	8	+	138	.032	.065	.070	.068
	9	+(Inactive)	0	.028	.027	.035	.034

[a] Xanthine oxidase preparation was inactivated by heating at 76° C. for 30 min., then added at the same rate as the active enzyme preparation.

were unexpected, because xanthine oxidase, an aerobic dehydrogenase, utilizes molecular oxygen as a hydrogen acceptor forming hydrogen peroxide. However, previous studies (2) indicated that hydrogen peroxide, when added to an enzyme-inactivated milk (76° C. for 30 min.), had no effect upon flavor development. Furthermore, the heated milk samples used in this study showed no measurable peroxidase activity; thus, the oxidation could not arise through the action of hydrogen peroxide (which would be expected to be formed in Sample 8) and peroxidase on an oxidizable substrate. Consequently, the effect exhibited by xanthine oxidase alone raises the question of the nature of the hydrogen acceptor involved.

Multiple enzyme systems vs. single enzyme in the development of oxidized flavor. Although there appeared to be a good correlation between xanthine oxidase activity and the development of spontaneous oxidized flavor, it was deemed necessary to consider the possibility of alternate pathways for flavor development. For example, peroxidase in the presence of hydrogen peroxide catalyzes the oxidation of many organic compounds, including ascorbic acid. Too, catalase in the presence of high substrate concentrations of low molecular weight alcohols and low peroxide concentration exhibits peroxidatic activity. Essentially, the same experimental design was used as in the foregoing experiments, except that lactoperoxidase was used as well as xanthine oxidase. The results of this study are presented in Table 2. It will be seen that the level of xanthine oxidase activity correlated well with flavor development; whereas, peroxidase activity had little or no effect on flavor development. Also, it was

TABLE 2

A study of the relationship of enzymes to oxidized flavor development as measured by the thiobarbituric acid method

Sample	Enzyme added	Xanthine oxidase activity	Peroxidase activity	Days			
				0	1	3	6
		(μl. O_2 uptake/hr/ml)	*(P.Z. units $\times 10^{-5}$)*	*(O.D.)*			
Raw	Control	51	104	.025	.025	.026	.025
	Xanthine oxidase	139	104	.035	.058	.063	.070
	Peroxidase	51	158	.033	.035	.040	.042
	Xanthine oxidase plus peroxidase	139	158	.034	.052	.060	.072
Pasteurized, 63° C. for 30 min.	Control	49	100	.026	.026	.027	.030
	Xanthine oxidase	138	100	.031	.056	.060	.065
	Peroxidase	49	160	.024	.027	.028	.031
	Xanthine oxidase plus peroxidase	138	160	.024	.056	.062	.070
Heated, 76° C. for 30 min.	Control	0	0	.025	.025	.025	.025
	Xanthine oxidase	143	0	.030	.050	.063	.072
	Peroxidase	0	154	.028	.028	.030	.032
	Xanthine oxidase plus peroxidase	143	154	.030	.052	.065	.070
	Xanthine oxidase plus peroxidase plus catalase[a]	143	154	.030	.055	.062	.068

[a] Added at the rate of 0.2 units/ml of milk.

noted that the addition of catalase to a heated milk sample which contained added xanthine oxidase and peroxidase had no effect on the flavor development. Thus, it was concluded that neither peroxidase nor catalase was involved in the direct pathway for development of spontaneous oxidized flavor.

The effect of inhibitors upon the development of spontaneous oxidized flavor. In view of the implications that only xanthine oxidase was involved in the development of spontaneously oxidized flavor, a study was made of the effect inhibitors of xanthine oxidase would have upon flavor development.

(a) Substrate inhibition. Many enzymes show inhibition by the substrate, but it appears that xanthine oxidase is exceptional, in that this inhibition occurs at unusually low substrate concentrations ($10^{-4}M$ or lower) (5). Hofstee (13), in studying the mechanism of substrate inhibition, reported that it was a non-competitive type of inhibition. In view of these facts, varying levels of hypoxanthine were added to milks that were adjusted for xanthine oxidase activity. Raw, pasteurized (63° C. for 30 min.), and enzyme-inactivated (76° C. for 30 min.) milks were used. The results of this study are presented in Table 3. It will be noted that an oxidized flavor developed in samples which were adjusted for xanthine oxidase activity and that did not contain added substrate. In contrast, the controls, unadjusted for xanthine oxidase activity, and the samples containing varying levels of hypoxanthine, failed to develop the flavor defect.

(b) Competitive inhibition. Among the effective inhibitors of xanthine oxidase are the pteridine derivatives (12, 14, 17). These inhibitors block the purine

TABLE 3
Effect of substrate upon development of oxidized flavor as measured by the thiobarbituric acid method

	Sample No.	Xanthine oxidase activity	Hypoxanthine added	O.D. at end of 6 days
		(μl. O_2 uptake/hr/ml)		
Raw	1 (Control)	103	0	.032
	2	131	0	.062
	3	131	4.4×10^{-7}	.030
	4	131	7.4×10^{-7}	.030
Pasteurized, 63° C.—30 min.	1 (Control)	94	0	.031
	2	131	0	.067
	3	131	4.4×10^{-7} *M*	.038
	4	131	7.4×10^{-7} *M*	.032
Heated, 76° C.—30 min.	1 (Control)	0	0	.032
	2	131	0	.070
	3	131	4.4×10^{-7} *M*	.040
	4	131	7.4×10^{-7} *M*	.037

and aldehyde dehydrogenating prosthetic groups of xanthine oxidase (16) and, thereby, lend themselves to a study of the role of xanthine oxidase in spontaneously oxidized flavor. Two inhibitors, 6-PA and folic acid, were used in this study. Herd milk was enzyme-inactivated by heating at 76° C. for 30 min. Then it was cooled and adjusted for xanthine oxidase activity. The milk was then subsampled; one portion served as the adjusted control and the others were treated according to the experimental design (Table 4). Flavor development was followed by use of the T.B.A. method. A summary of the data is presented in Table 4. The inhibitors were effective in preventing the development of the flavor defect and, hence, were viewed as inhibiting at the dehydrogenase site. It will be noted that 6-PA exerted a greater inhibitory effect than did folic acid. This is in agreement with an observation made by Kalckar *et al.* (14), who reported that (6-PA) on a molar basis is 200-400 times more active as an inhibitor of xanthine oxidase than is folic acid.

TABLE 4
Effect of inhibitors of xanthine oxidase upon the development of oxidized flavor as measured by the thiobarbituric acid method

Type of inhibition	Sample	Inhibitor added	Concentration	Days 0	1	3	6
				(O.D)			
	Unadjusted control[a]	0	0	0.028	0.030	0.030	0.032
	Adjusted control[b]	0	0	0.030	0.052	0.062	0.070
Competitive	1	6-PA	5×10^{-6} *M*	0.030	0.034	0.038	0.042
	2	Folic Acid	10^{-4} *M*	0.030	0.048	0.057	0.065
	3	Folic Acid	10^{-3} *M*	0.028	0.038	0.045	0.053
	4	Folic Acid	2×10^{-3} *M*	0.032	0.030	0.030	0.030
Noncompetitive	5	PCMB	10^{-4} *M*	0.027	0.045	0.058	0.065
	6	PCMB	10^{-3} *M*	0.030	0.032	0.035	0.035

[a] Xanthine oxidase activity = 75 units.
[b] Xanthine oxidase activity = 138 units.

(c) Noncompetitive inhibition. Although the above study on substrate inhibition was a study dealing essentially with a noncompetitive type of inhibition, it was deemed necessary to consider another noncompetitive inhibitor (e.g., PCMB). A summary of the data is shown in Table 4. This inhibitor, likewise, was effective in preventing flavor developmnt.

DISCUSSION

The results obtained from the above studies show: (1) The development of spontaneously oxidized flavor could be correlated with xanthine oxidase activity, (2) a single enzyme rather than a multiple enzyme system was involved in the flavor defect, and (3) known inhibitors of xanthine oxidase were effective in preventing flavor development. Thus, it was concluded that the occurrence of a spontaneously oxidized flavor in milk was dependent upon a high level of xanthine oxidase activity. Furthermore, it was concluded that the use of an enzyme inhibitor (e.g., a pteridine derivative) would enable one to control the occurrence of spontaneously oxidized flavor.

Xanthine oxidase is usually considered as an aerobic dehydrogenase, i.e., it reacts directly without the intervention of intermediary hydrogen carriers. The results obtained from the study of multiple enzymes as compared to a single enzyme indicated that only a single enzyme was involved. These observations, coupled with the observation that the flavor defect occurred in the enzyme-inactivated milk to which a crude preparation of xanthine oxidase had been added (Sample 8—Table 1), gave rise to the question of the role of hydrogen peroxide formed because of xanthine oxidase activity. In an earlier study (2), it was reported that the addition of preformed hydrogen peroxide to enzyme-inactivated milk had no effect on the development of oxidized flavor. Conversely, hydrogen peroxide when added to susceptible raw milk at low concentration (25 γ/ml) gave rise to a pronounced oxidized flavor; whereas, a higher concentration (50 γ/ml) inhibited flavor development. Chance (4), in discussing hydrogen peroxide reactions with hemoproteins, suggested that small concentrations of hydrogen peroxide may be disposed of by peroxidatic enzyme action and larger concentrations may bring into play the catalytic action. Keilin and Hartree (15) reported that peroxidatic enzyme action was dependent on a continuous supply of hydrogen peroxide, such as generated by a dehydrogenase. Therefore, since hydrogen peroxide was effective only in the presence of an enzyme, and only in a limited concentration, it would seem reasonable to conclude that xanthine oxidase exerted a peroxidatic action.

The peroxidatic action of xanthine oxidase may be explained on the basis of a free radical mechanism. One of the prevalent views regarding oxidation-reduction reactions in aqueous solutions is that the reactions involve free radicals and proceed by means of a chain mechanism. Xanthine oxidase is a metallo-flavoprotein enzyme which contains iron and molybdenum as the metal ions. These ions are essential to the action of the enzyme and are reversibly oxidized and reduced during the normal course of the enzymatic reaction. Recently, Fridovich and Handler (9) have proposed a structure for the active site of xanthine oxidase

which includes a ferric-mercaptide group as an essential part. In the normal course of reaction, electrons pass from the substrate to the first flavin and, thence, via the ferric-mercaptide groups, which are single electron acceptors; then, to the second flavin, from which they may pass to oxygen and other acceptors. Thus, the chief role of the iron ion is to make possible a very rapid electron transfer, i.e., the reversible change between the divalent and trivalent state. It is thought this explanation for the active site of xanthine oxidase can be used to explain the peroxidatic action of the enzyme by the following scheme:

(a) $1\,e^- + H^+ \longrightarrow E\,Fe^{+++} \longrightarrow HE\,Fe^{++}$

(b) $HE\,Fe^{++} + O_2 \longrightarrow E\,Fe^{+++} + HO_2$

(c) $1\,e^- + H^+ + HO_2 \longrightarrow H_2O_2$

(d) HO_2 + oxidizable substrate $\longrightarrow$ oxidized flavor

As indicated in the hypothesis above, the uncomplicated oxidation of a substrate by xanthine oxidase results in the formation of hydrogen peroxide (a), (b), and (c); whereas, the intervention of a substance oxidizable by the transitory radical, HO_2, would result in reaction (d). Thus, according to this view, an intermediate product of hydrogen peroxide formation is the oxidant rather than hydrogen peroxide per se. Consequently, there is no need for a peroxidase in the system. The results presented herein fit this hypothesis. Furthermore, this hypothesis is substantiated by the work reported by Green and Mazur (10), in which they describe a similar reaction involving xanthine oxidase.

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WHEY PRESERVATION BY HYDROGEN PEROXIDE

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SUMMARY

Concentrations of 0.02% H_2O_2 in cheese whey exerted a preservative effect for as long as ten days. Bacterial plate counts were made of peroxide-preserved wheys of pH 4.9, 5.9, and 6.9 kept at 25, 37, and 50° C. According to these data, H_2O_2 addition shortly after separation of the whey from curd is recommended. Peroxide addition to grossly contaminated wheys of 2.8×10^7 microorganisms per milliliter resulted in a 97% bacterial kill within 1 hr. The 0.02% H_2O_2 concentration was relatively ineffective against greater numbers of bacteria. *Saccharomyces fragilis* yeast was propagated in peroxide-preserved whey from which the peroxide had been removed by the action of catalase. Results were comparable to a control fermentation on untreated fresh whey.

Enormous quantities of liquid whey from cheese are available for utilization. The United States cheese-making industry alone produces over twelve billion pounds of whey annually (8). Although it is economically impractical to consider utilization of this entire amount, increased uses for a greater proportion of the whey are possible. Suggested outlets are many (3), ranging from returning liquid whey to the farms for livestock feeding to using it as a supplemented medium in the fermentation industry.

Liquid whey remaining from the cheese-making process is composed of about 4 to 5% lactose and 0.9% protein, and constitutes a good bacterial substrate. Bacterial decomposition of this whey is a problem when storage or transportation is necessary. The use of a relatively harmless preservative may be advisable, especially if the whey is to be used for microbiological processes. In addition to being nontoxic, the preservative should be inexpensive, nonreactive with whey components, and be easily destroyed or removed when necessary.

Hydrogen peroxide has long been recommended as a dairy preservative in tropical countries. Excellent comprehensive reviews on the subject have been prepared by Rosell (10) and Lück (5). This strong and active germicide has been found acceptable in preventing souring of milk during transport and in preserving milk for cheese-making. In H_2O_2 milk-preservation methods practiced abroad, an addition of catalase is often made before the milk is ready for human consumption. This enzyme has the property of splitting H_2O_2 into its breakdown products, water and oxygen. Its use has been studied by Curran, Evans, and Leviton (2) in sporicidal action of hydrogen peroxide.

The widespread and sanctioned use of H_2O_2 as a milk preservative in some countries (9) suggested laboratory investigations into its applicability as a preservative for liquid whey. Previous studies in this area have been made by Plöttner (7), who used 0.015 to 0.03% H_2O_2 (by weight) in the preservation of

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raw sweet whey and removed H_2O_2 residues by heat precipitation of the proteins or by addition of yeast. Results of our laboratory investigations on the peroxide-preservation of whey and its subsequent use for yeast growth are given in this report.

MATERIALS USED

The laboratory studies were made on liquid whey obtained from a local cottage cheese–manufacturing concern. The whey as it was received from the plant had a pH of 4.5 and temperature up to 60° C. It was transported to the laboratory in milk cans and placed in a refrigerated room at 4° C. immediately upon arrival. The insoluble proteins settled readily, and only the supernatant fluid was used for these studies.

Reagent-grade hydrogen peroxide of 28–30% purity was employed as the preservative, but data are reported on the basis of 100% purity H_2O_2. Exact composition was determined and frequently checked by Kingzett's iodine-thiosulfate titration method (11). The H_2O_2 was kept at 4° C. to prevent unnecessary deterioration due to room temperatures.

The inoculum used for testing preservation action was propagated in the laboratory at room temperature throughout this series of experiments. Sporadic feedings of both dried skimmilk and liquid whey were made to maintain its vigor. The culture consisted of an aerated sludge mixed with commercial milk that had soured. No attempt was made to identify the organisms in the mixture, except for testing their ability to attack milk and whey. An earlier investigation and isolation of the aerated sludge organisms, however, showed the predominance of *Alcaligenes*, *Flavobacterium*, *Micrococcus*, *Pseudomonas*, *Bacillus*, and *Bacterium* genera (4).

Hydrogen peroxide destruction was catalyzed by solutions of a commercial catalase preparation, Armalase A-100, a purified preparation of the enzyme manufactured by Armour Company.[2] Its potency is 100 Keil units per milliliter, one unit being the quantity needed to decompose 1 g. of 100% H_2O_2 in 10 min. at 25° C., in an inert atmosphere of CO_2 or N_2 (1).

STUDIES ON MINIMUM EFFECTIVE H_2O_2 CONCENTRATIONS

Preliminary studies were made on 50-ml. quantities of whey placed in sterile Erlenmeyer flasks having cotton plugs. Varying protective amounts of H_2O_2 were added. Half of the flasks were inoculated with the test organisms (50,000 organisms per flask); the remaining flasks were to contain only indigenous whey organisms. The flasks were held at room temperature and examined daily for physical changes in appearances. Bacterial growth was detected by transferring a loopful of the whey to sterile whey-nutrient broth solution and observing turbidity after incubation at 30° C. Results of these tests are given in Table 1 and point to 0.020% H_2O_2 as the effective level of protection. For ten days of the experiment, the broth tubes gave like turbidity readings, showing that the peroxide

[2] It is not implied that the USDA recommends the above company or its product to the possible exclusion of others in the same business.

TABLE 1

Bactericidal effect of H_2O_2 as determined by a sterility test procedure

H_2O_2 in whey	Age of wheys (in days)					
	1–3		4–7		8–10	
	Bacterial growth in sterile broth					
	I	U	I	U	I	U
(%)						
0	+	+	+	+	+	+
.010	+	−	+	−	+	−
.015	+	−	−	−	+	−
.020	+	−	−	−	−	−
.025	−	−	−	−	−	−
.030	−	−	−	−	−	−

I = Inoculated whey; U = uninoculated whey.

had checked further bacterial growth. On the 11th day, tests for residual H_2O_2 showed traces still present in all wheys. Excess quantities of sterile (Seitz-filtered) catalase were added on the 13th day to destroy the H_2O_2 and to leave the whey susceptible to possible bacterial attack. The next day, the whey solutions were reinoculated with the test organisms and reincubated at 30° C. All the flasks showed bacterial turbidity, proving that the catalase had removed all the H_2O_2.

Table 2 gives results of a test in which 1-liter amounts of whey from the cheese vat were held at room temperature (22–25° C.) in glass flasks covered with beakers. These received no supplementary organisms. Standard bacterial plate counts were made on nutrient agar incubated at 37° C. for 48 hr., as used in water analyses. Only the unprotected whey showed evidence of contamination. After 2 wk., a dense growth of a fungus, presumably *Aspergillus niger*, had appeared on the surface of all three flasks. However, for six days at room temperature, these commercial wheys had been satisfactorily protected from bacterial growth.

TEMPERATURE AND PH EFFECTS ON THE BACTERIAL ACTION OF H_2O_2

The bactericidal action of 0.02% H_2O_2 concentration at different temperatures was investigated. One hundred–milliliter quantities of refrigerated cheese whey were put into sterile bottles and equilibrated at 50, 37, or 25° C. Sufficient bacterial inoculum obtained from well-aerated sludge culture was added to give 1,000 organisms per milliliter of whey. One milliliter of 2% H_2O_2 was added, resulting in a 0.02% concentration in the final whey. At 10-min. intervals,

TABLE 2

Preservation of fresh whey by H_2O_2 as measured by bacterial plate counts[a]

H_2O_2	Bacterial count per milliliter whey		
	First day	Fourth day	Sixth day
(%)			
0	51	2,500	650,000
.020	0	0	0
.025	0	0	0

[a] 48-Hr. growth on nutrient agar at 37° C.

samples were removed for plate count determinations on nutrient agar medium. The bacterial colonies were counted after 48 hr. of incubation at 37° C.

Figure 1 shows the destruction attained at the three temperatures, as plotted on a semilog scale. Most rapid killing was observed at 50° C. Here, the high initial temperature alone had reduced the bacterial count from over 1,000 organisms per milliliter to 230. Within 5 min., 75% of the remaining organisms was destroyed by the H_2O_2. At 37 and 25° C., similar, but not such marked, effects of H_2O_2 were noted. Seventy-five per cent destruction was obtained within 13 min. at 37° C. and within 24 min. at 25° C. At none of these temperatures did the 0.02% H_2O_2 concentration achieve 100% destruction.

Table 3 summarizes the combined effects of temperature, pH, and 0.02% H_2O_2 on the viability of organisms in whey solutions. Natural whey of pH 4.9 and wheys adjusted to pH 5.9 and 6.9 with NaOH were heated to indicated temperatures and inoculated to contain 1,000 organisms per milliliter. After removing the samples for initial bacterial counts, peroxide was added. Samples were removed after 10 and 20 min. for bacterial counts. High temperatures and low pH had an immediate destructive effect, particularly at pH 4.9 and 50° C., destroying over 99% of the original organisms. The 25 remaining colonies were almost all yeasts and fungi, forms somewhat refractory to peroxide treatment as indicated by the 68% kill figure. In spite of the different beginning temperatures, pH's, and variations in viable organisms, maximum destruction occurred in all cases within 10 min. after peroxide addition. Samples taken even after an exposure of 1 hr. to peroxide showed practically no further destruction of the microorganisms.

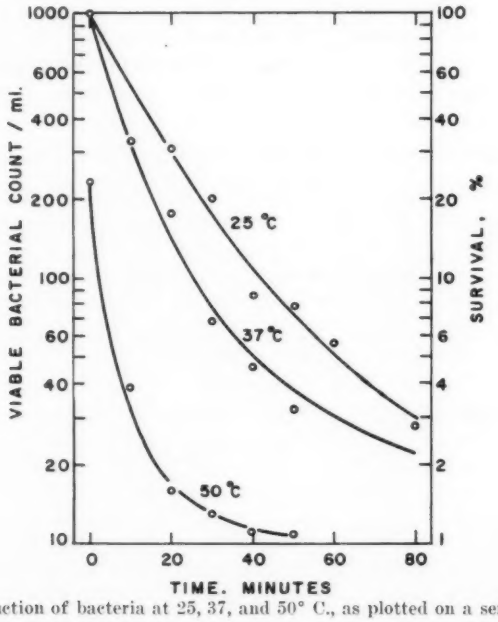

FIG. 1. Destruction of bacteria at 25, 37, and 50° C., as plotted on a semilog scale.

TABLE 3

Bacterial destruction in inoculated whey treated with 0.02% H_2O_2 as affected by temperature and pH

Time (min.)	25° C. (No/ml)[a]	(%)	37° C. (No/ml)[a]	(%)	50° C. (No/ml)[a]	(%)
			pH 4.9			
0	537		817		25	
10	35	(93)	24	(97)	8	(68)
20	25	(95)	18	(97)	8	(68)
			pH 5.9			
0	842		931		349	
10	56	(93)	35	(96)	9	(97)
20	34	(96)	32	(96)	6	(98)
			pH 6.9			
0	1,000		1,035		573	
10	181	(82)	61	(94)	8	(98)
20	61	(94)	64	(94)	4	(99)

Figures in parentheses represent percentage of bacteria killed, based on actual zero-hour figures. Percentage kill would be greater if based on initial inoculation of 1,000 organisms per milliliter.

[a] Bacterial plate counts on nutrient agar incubated at 37° C. for 48 hr.

BACTERICIDAL POTENCY OF 0.02% H_2O_2

Test organisms were used in these laboratory studies in concentrations of 1,000 organisms per milliliter of whey for these reasons: first, heated wheys received from the cheese manufacturer numbered from zero to about 400 bacteria per milliliter; secondly, 1,000 bacteria per milliliter furnished a convenient number for making bacterial plate counts without further dilution. The destructive effect of 0.02% H_2O_2 on large numbers of bacteria as may occur in contaminated whey remained to be determined. For this work, a bacterial culture was propagated from a contaminated commercial yoghurt preparation and from soured milk, and was considered representative of dairy contaminants. Bacteria numbered 2.8×10^9 in the undiluted culture. Five tenfold dilutions were made in whey. Hydrogen peroxide was added to each flask to give a 0.02% final concentration.

Table 4 presents the viable plate counts and per cent destruction achieved by 0.02% H_2O_2 acting on ranges of bacteria from 10^9 to 10^4. This concentration effected 97% destruction of 2.8×10^7 bacteria after 1 hr. of contact. After 24 hr., a 99% kill was measured. Apparently, the peroxide effect is completed within the 1st hr., as seen also with the other dilutions. The lower percentage of destruc-

TABLE 4

Destruction of bacteria in contaminated whey by 0.02% H_2O_2

Original count[a]	After 1 hr.	Kill	After 24 hr.	Kill
(per ml.)		(%)		(%)
2.8×10^9	3.6×10^9	0	2.7×10^9	Negligible
2.8×10^8	1.8×10^8	36	2.1×10^8	25
2.8×10^7	0.087×10^7	97	0.023×10^7	99
2.8×10^6	0.0085×10^6	99+	< 10	99+
2.8×10^5	0.00035×10^5	99+	< 10	99+
2.8×10^4	0	100+	< 10	99+

[a] Bacterial plate counts on nutrient agar incubated at 37° C. for 48 hr.

tion that occurred with the higher concentration of cells may be explained by the probable presence of catalase, elaborated by greater numbers of catalase-producing organisms. The effect of the small amount of peroxide could be quickly negated, and the bacteria would continue multiplying.

PROPAGATION OF YEAST IN PEROXIDE-PRESERVED WHEY

The effect of peroxide treatment on whey used for subsequent propagation or fermentation was investigated. One liter of liquid whey was treated with 0.02% H_2O_2 and held uncovered at room temperature (24° C.) for 24 hr. Excess catalase was added (30 units of Armalase) and allowed to stand for 30 min. Spot-testing with potassium iodide showed that all the peroxide was gone. This whey was used as part of a growth medium for the propagation of the yeast, *Saccharomyces fragilis* (12). A control fermentation with untreated whey also was run. Data compiled during these fermentations are compared in Table 5. After 3 hr., both fermentations were terminated. The harvest was about the same, and only minor differences were observed between the two fermentations.

DISCUSSION

The 0.02% H_2O_2 concentration showed both a bactericidal and bacteriostatic action against 1,000 bacteria added per milliliter of whey, and inhibited bacterial growth for as long as ten days. Traces of peroxide were easily and completely removed by the enzymatic action of subsequently added catalase, leaving the solution again susceptible to microbial attack. Satisfactory removal was demonstrated also by the successful propagation of yeast in cottage cheese whey preserved with peroxide.

Optimum conditions for peroxide treatment were suggested in the study on temperature and pH effects. Waste whey is available from cottage cheese–making operations at temperatures up to 60° C. at a pH of about 4.5 These conditions are not favorable for bacterial growth. On cooling to only 37° C., however, this whey becomes increasingly attractive to bacteria. Peroxide addition is advisable, therefore, as soon as the whey is collected.

The rate and degree of bacterial destruction by peroxide is dependent on the numbers and types of organisms and on the concentration of peroxide used. The bacterial mixture used in our experiments constituted a stringent test. This culture contained bacteria, yeast, and fungi ordinarily associated with aerobic dairy waste treatment (4). It should be noted that although 0.02% H_2O_2 accomplished up to 98% destruction of these test organisms within as little as 10 min. (at 50° C. and pH 6.7), complete destruction of all growth was

TABLE 5

Propagation of *Saccharomyces fragilis* in fresh and 0.02% H_2O_2-treated wheys

Time	Yeast count		Dry weight		Lactose		pH	
	Fresh	Treated	Fresh	Treated	Fresh	Treated	Fresh	Treated
(hr.)	*($\times 10^6$/ml)*		*(mg/ml)*		*(mg/ml)*			
0	1,450	2,010	12.96	13.14	39.6	40.2	5.5	5.5
2	3,540	3,460	27.99	28.10	17.6	19.6	5.8	5.95
3	5,380	4,380	36.32	36.04	2.2	3.5	5.2	4.85

not attained. After the rapid initial killing, the peroxide effect seemed to slacken. In other tests with this same mixture of organisms, even 24 hr. of exposure to 0.02% H_2O_2 did not show 100% reduction in bacterial count. Varying degrees of resistance and indifference to the preservative are expected in such a mixture of microorganism. The susceptible species, primarily the Gram-negative forms, are annihilated; others, such as the Gram-positive rods, are unaffected and continue to proliferate. More potent concentrations of peroxide, although more destructive, were observed to precipitate and modify the whey proteins.

The bactericidal limitations of a 0.02% final concentration of H_2O_2 on milk-souring types of organisms were investigated. Within 1 hr., a 97% kill of 2.8×10^7 bacteria was effected, a remarkable achievement considering that this number of bacteria represents a grossly contaminated material. Studies showing 100% destruction of milk microorganisms have been reported by Nambudripad (6), who found 16 hr. of exposure to 0.05% H_2O_2 at 37° C. necessary for full destruction of *Bacillus megatherium* in broth solutions. This contrasted greatly to the much shorter time of 30, 40, and 45 min. required for 100% killing of *Aerobacter aerogenes*, *Alcaligenes viscosus*, and *Escherichia coli*, respectively, all Gram-negative and peroxide-sensitive forms.

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WHEY AS A SOURCE OF PLANT NUTRIENTS AND ITS EFFECT ON THE SOIL[1]

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SUMMARY

The possibility of the use of whey as a source of plant nutrients was investigated through laboratory analysis and field studies. A ton of whey was found to contain about one dollar's worth of nitrogen, phosphorus, and potassium, plus significant amounts of sodium, calcium, magnesium, and chlorides.

Whey applications do not have a detrimental effect on the pH of soils which are well limed and near neutral. However, whey added to soils with a pH of 5.0–5.5 may temporarily increase the soil acidity to a point which is injurious to plant growth.

Alfalfa will tolerate a limited amount of whey but does not benefit much from it. About one-half acre inch per week is the maximum quantity tolerable. Grasses appear more tolerant, and whey applications increased the growth of bluegrass, especially during the second growing season. This delayed benefit seems due to the slow breakdown of nitrogen compounds in the whey.

Whey benefits soil aggregation, with the amount of aggregation being dependent on the amount of whey added. However, exceedingly large applications may result in a reduction in the formation of aggregates.

Approximately 11 billion pounds of whey are produced annually in the United States. Of this total, nearly one-third occurs during the 2 or 3 mo. of the year when its production is at a maximum and its use as animal feed is at a minimum. Not only does this waste result in the loss of considerable quantities of valuable animal and plant food, but also causes a severe disposal problem.

Few research workers have studied the possibility of using whey as a soil amendment. However, in 1923, Berry (2) presented a thorough study on the utilization of whey in Scotland. In a greenhouse experiment using infertile sandy soil, he found that whey had a beneficial effect on the growth of oats. Where whey was applied on pasture at the rate of 2¼ tons per acre per day from June until September for several years, the only apparent effect was greater growth of the coarser grasses. Berry concluded that whey was beneficial to the soil but that hauling costs would probably be too high to make it profitable in most cases.

In a study of the nutritive value of whey applied to plants grown in the greenhouse on Miami silt loam, Cain (3) found that oats which received a single 100-ton-per-acre application of whey at time of planting were initially stunted, but recovered and gave a greater yield than those which did not receive whey. On a treatment that received continuous applications of whey which totaled 200 tons per acre, over a 2.5-mo. period, the oats were stunted during the early stages of growth and recovered in the later stages only when the whey applications

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were reduced. It appeared that even though whey was beneficial, in the long run very heavy and continous applications would temporarily inhibit plant growth. In a field experiment, Cain (3) applied whey on bluegrass and quackgrass sod in an effort to confirm the results he had obtained in the greenhouse. Although he concluded an increase in yield could be seen for each addition of whey, he felt that with the late fall or early spring applications, little nitrogen in the whey is released to the plants during the early part of the following growing season.

This project involved the establishment of a maximum level at which whey can be applied to field crop and the effects on crop growth and soil structure.

RESULTS AND DISCUSSION

Whey composition. Whey samples were collected at seven different times from April to November, 1957, at the Madison Milk Producers Cooperative Dairy, Madison, Wisconsin. The samples were largely a composite of whey from Swiss and American cheese, and a small amount from cottage cheese. Each sample was run in triplicate.

Method of analysis. An aliquot of 50 ml. of whey was evaporated to dryness on a 95° C. hot plate. The samples were then placed in a muffle furnace and ignited at 500° C. for 3 hr. Calcium, magnesium, potassium, and sodium were determined by extracting with .4 *N* HCl and analyzing on the Beckman Flame Photometer. Phosphorus was analyzed on the same sample, using the vanadomolybdate yellow color method (5). Nitrogen was analyzed on a 5-ml. aliquot by the Kjeldahl procedure as modified by Jackson (5). Chlorides were determined on a 10-ml. aliquot of the raw whey, which was brought to 50 ml. in volume with distilled water. About 0.5 ml. of saturated potassium chromate was used as an indicator and the sample titrated with 0.282 *N* silver nitrate (6).

The analysis given in Table 1 indicates that whey has sufficient quantities of the elements used in plant growth to be of value as a soil amendment. The nitrogen, phosphorus, and potassium in one ton of whey is worth about one dollar, based on current fertilizer prices.

TABLE 1

Analysis of separated whey from Swiss and American cheese, collected at the Madison Milk Producers Cooperative Dairy from April to November, 1957

Sample No.	Elements						
	Ca	Mg	Na	K	P	N	Cl
	(p.p.m)						
1	300	50	280	1,630	400	1,400	1,000
2	350	50	650	1,750	450	1,200	1,200
3	300	60	400	1,700	450	1,200	1,150
4	300	50	480	1,800	500	1,500	1,150
5	300	50	300	1,600	500	1,350	1,000
6	350	60	800	1,600	450	1,400	1,250
7	300	50	400	1,650	400	1,500	1,200
Average	327	53	471	1,679	450	1,364	1,136

Sodium and nitrogen appear to be the two elements with the greatest amount of variation. This variation may be due to a particular type of process being used in cheese manufacture. The quantity of nitrogen may vary, depending on the amount of protein and other nitrogen-containing milk constituents left in the whey. The sodium content varies with the amount and type of salting process being used.

Effect of whey on soil pH. It has long been recognized that soil pH is one of the important factors affecting plant growth. Therefore, the effect of an acid whey solution on the soil pH was determined.

Two soil types were used, a Miami silt loam (forested soil derived from limestone glacial till), with an original pH of 6.8 and a Spencer silt loam (forested soil derived from granitic glacial till), with an original pH of 5.2.

For each type of soil, 560 g. was placed in a 600-ml. beaker and then brought to field moisture capacity by adding a combination of whey and water. The proportion of whey and water was varied to represent whey application ranging from 0 to 160 tons per acre. The pH of the whey was 4.0 at the time of application. Following the application of whey, pH readings were taken at the time intervals shown in Table 2.

TABLE 2

Soil pH resulting from various rates of application of whey on Miami and Spencer silt loam soils after various time intervals

Hours after initial application	Spencer silt loam[a]				Miami silt loam[b]			
	Whey applied in tons per acre							
	0	40	80	160	0	40	80	160
2	5.22	4.75	4.65	4.56	6.74	6.41	6.20	5.90
4	5.21	4.84	4.69	4.57	6.73	6.25	6.32	5.95
8	5.08	4.75	4.65	4.55	6.78	6.18	6.28	5.59
12	5.23	4.86	4.75	4.57	6.78	5.87	6.15	5.60
24	5.28	5.02	4.86	4.61	6.80	5.95	6.25	5.45
48	5.43	5.19	5.07	4.90	7.08	7.00	6.68	5.65
72	5.34	5.29	5.16	4.97	6.81	7.10	7.10	5.56
98	5.42	5.32	5.15	4.95	6.82	7.38	7.38	6.10
192	5.49	5.44	5.25	5.20	6.75	6.95	6.95	7.10

[a] Initial soil pH = 5.2.
[b] Initial soil pH = 6.8.
[c] pH of whey at time of application, 4.0.

As indicated in Table 2, the drop in soil pH and the length of time that the decrease persisted appeared to be dependent upon the quantity of whey added. The magnitude of the change appeared to be nearly equal in both soils. In the neutral Miami soil, the addition of whey does not cause the soil pH to drop to a point which would be injurious to plants. However, in the strongly acid Spencer soil the addition of whey may temporarily lower the pH to a point injurious to the plant metabolism.

After the whey was added to the Miami soil, there was a gradual increase in soil pH which eventually exceeded the pH of the control. This may be explained by decomposition of nitrogenous compounds releasing ammonia.

Field study on bluegrass. The first experiment was designed to indicate the residual effect and fertility value of whey on bluegrass sod.

Original whey and/or fertilizer applications to the experimental plots were begun by Cain (3) on Miami silt loam soil in November, 1955, and April, 1956. The effects of the whey and fertilizers on the growth of bluegrass during the first and second growing season following application are reported in Table 3.

There was no difference in bluegrass yields due to fall versus spring applications of whey and/or fertilizer. However, there was a significant increase in bluegrass yield for the 80 tons per acre of whey, or 40 tons of whey fortified with 240 and 1,040 lb. per acre of nitrogen supplied as ammonium hydroxide. The results also indicate that during the two growing seasons following the application of 80 tons of whey, bluegrass yields were comparable to the yields from the application of 930 lb. of 12-12-12, or about 100 lb. of nitrogen in the form of ammonium nitrate, urea, sodium nitrate, or solution nitrogen. Plots which received only nitrogen gave a good yield the first growing season, which also carried over into the second growing season. This indicates that not all the fertility value of the whey is immediately available after application, but that appreciable benefits may be derived during the second growing season, as well.

Field study on alfalfa. The boundaries of the alfalfa field plots were corrugated steel (9 in. by 12 ft.) driven 5 in. into the ground. Thrice-weekly applications, usually on alternate days, applied from 64 to 384 tons of whey or water per acre per week. The control treatment received 128 tons of water per week to approximate soil moisture conditions of the whey plots. To compare the plant nutrients in the whey with those in fertilizers, 1,000 lb. of 10-10-10 per acre was broadcast previous to any liquid applications. In one treatment, the carbon-nitrogen ratio of the whey was lowered from about 35:1 to less than 20:1 by adding ammonium nitrate. This brought the carbon-nitrogen ratio more in

TABLE 3

Residual effect of whey and/or fertilizer applications on bluegrass the first and second growing seasons after application. Bellefontaine silt loam, Gugle Farm, Madison, Wisconsin

Treatment per acre	First growing season yield[a]	Second growing season yield[b]	Average
Control	2,225[c]	2,029[c]	2,127[c]
20 Tons whey	2,295	2,459	2,377
40 Tons whey	2,830	2,861	2,846
80 Tons whey	3,310	3,821	3,566**
40 Tons whey plus 80 lb/acre of N (NH_4OH)	2,880	3,069	2,975
40 Tons whey plus 240 lb/acre of N (NH_4OH)	4,020	3,580	3,800**
40 Tons whey plus 1,040 lb/acre of N (NH_4OH)	2,380	3,794	3,087**
930 lb. 12-12-12	4,460	2,661	3,560**
112 lb. nitrogen as NH_4OH	2,800	2,075	2,438
700 lb. $NaNO_3$	4,200	2,695	3,448*
267 lb. as urea	4,030	2,398	3,214*
340 lb. as NH_4NO_3	4,530	2,673	3,602**
350 of "Uran"	4,630	2,560	3,595**

* Least significant difference at the 5% level—1,040 lb.
** Least significant difference at the 1% level—1,390 lb.
[a] Harvested in June, 1956.
[b] Harvested in June, 1957.
[c] All yields given in pounds dry matter per acre.

balance with the 10:1 soil carbon-nitrogen ratio. During the experiment, about 600 lb. of ammonium nitrate and 180 tons of whey were applied to this treatment. The results of these applications are shown in Table 4.

The soil pH was 6.7 and the alfalfa was about 6 in. high when the first applications were made on July 5, 1957. When the plots were harvested at the end of the 3rd wk., most of the plants were dead on plots receiving 128, 192, and 384 tons per acre per week. Strong odors had resulted from the 384-ton treatment, whereas only slight odors could be noticed from the rest. Only plants receiving 64 tons of whey per acre per week appeared normal, with only a slight loss and yellowing of some of the leaves near the soil surface. The pH of the plow layer was not appreciably affected by any of the treatments. The tissue samples were dried, weighed, and yields calculated on the dry weight basis as shown in Table 4.

Due to a whey shortage and an extended dry period, resumption of the treatments was impossible and the regrowth was slow except for a luxuriant growth of volunteer pigeon grass on the 128- and 192-ton treatments. No growth of any kind occurred on the 384-ton treatment.

TABLE 4

Influence of whey, water, and/or fertilizer on growth of second-crop alfalfa, and formation of soil aggregates. University Hill Farm, Madison, Wisconsin

(Whey applied July 5 through July 24, 1957)

Liquid and amount applied per wk.	Total liquid applied	Fertilizer applied	Yield-lb. dry matter per acre[a]	Aggregates in each size fraction[c]			
				2.0 mm.	1.0 mm.	0.5 mm.	0.25 mm.
(tons/acre)	*(tons/acre)*			(%)			
128 Water	294	None	3,052	3.89	6.60	14.80	34.72
64 Water	181	1,000 lb. 10-10-10	3,624	4.81	7.49	15.36	34.18
64 Whey	181	None	2,622	9.36	14.0	23.82	42.90
64 Whey	181	72 lb. NH_4NO_3 weekly	2,857	10.08	15.27	25.83	44.58
128 Whey	378	None	[b]	12.30	19.81	33.00	51.81
192 Whey	512	None	[b]	10.00	16.11	28.24	46.30
384 Whey	768	None	[b]	5.99	10.59	22.55	40.97

[a] Average of four replicates.
[b] Most plants dead at end of treatment.
[c] Sampled May 7, 1958.

The yield of alfalfa was reduced with the whey treatment, in spite of the addition of the ammonium nitrate. Therefore, it appears that alfalfa can tolerate only about 0.5 acre inch per week without harm, and that quantities in excess of this will result in killing and stunting of many of the alfalfa plants. This may be the result of the extreme demands put on the oxygen supply of the soil when large quantities of easily decomposable organic matter are added. However, the heavy growth of the pigeon grass on the 128- and 192-ton treatments indicates that a shallow root system would be more satisfactory. It appears that grasses are more tolerant to large whey applications, and that whey will have residual benefits on bluegrass even during the second growing season.

While conducting a greenhouse study on soil aggregation, Chesters (4) used various materials such as whey, sucrose, corn stover, cow manure, sawdust, and

various peats. He found that a single application of 150 tons per acre of whey gave the maximum per cent of soil aggregates over 0.25 mm. in diameter. Soil aggregates may be defined as naturally occurring clusters of soil particles which promote soil tilth, drainage, and aeration, and increase water-holding capacity and reduce erosion.

To obtain information under field conditions, soil aggregation analyses were made on samples taken about 10 mo. after whey applications were made. The analysis was made by using the wet sieving method devised by Yoder (7) and modified by van Bavel (1), to determine the percentage of water stable aggregates which is indicative of desirable soil structure. The results are given in Table 4 and indicate that all levels of whey application helped to form water-stable aggregates. The number and size of aggregates increased with each increment of whey to the 192-ton treatment, after which there was a decrease approximately to the level of the 64-ton treatment. At the time of sampling for aggregate analysis (May, 1958), no regrowth had occurred on the 384-ton treatment; whereas, the remainder had a mixture of alfalfa and grass. The lack of cover on the 384-ton plots may well have caused a breakdown of the aggregates during the winter freezing and thawing and the rainfall impact during the spring rains.

CONCLUSION

The present study indicates that whey can improve soil structure and provide plant nutrients, but it appears that feeding to animals would accomplish more complete utilization of whey solids. However, at periods of maximum production, when adequate disposal means are unavailable, the application of whey to soil can benefit the crops and soil, where care is exercised in its application.

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GENETIC AND ENVIRONMENTAL INFLUENCES UPON SOLIDS-NOT-FAT CONTENT OF COW'S MILK[1]

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SUMMARY

Monthly composite milk samples from the Holstein and Guernsey herds of the New Jersey Agricultural Experiment Station were taken from July, 1953, to February, 1958. Solids-not-fat (SNF) content was determined by formulas based on specific gravity (Watson Pattern Lactometer) and fat percentage (Babcock fat test). A study of 4,772 samples from 222 cows revealed no specific yearly trends in SNF content, although 1954 was significantly lower with Holsteins, and 1956 significantly higher with Guernseys, than the averages of the remaining years. Month effects were significant and similar for both breeds. SNF content was high in December and January and low in June and July. Although stage-of-lactation effects were more extreme on Holsteins, SNF content of both breeds was high shortly after parturition, dropped to a lactation low at 40 to 60 days, increased very slowly to 6 mo., and then increased rapidly to 10 mo. Pregnancy effects were noticeable at the 4th or 5th mo. of gestation, and accounted for most of the SNF increase in the latter stages of lactation. Two types of lactation averages were calculated: (1) based on all tests from parturition to 305 days, and (2) based on the second through sixth tests. Records from 182 cows by 50 sires were studied. SNF content of 310 Holstein records averaged (1) 8.48 and (2) 8.42%; 110 Guernsey records averaged (1) 8.95 and (2) 8.93%, respectively. SNF content decreased with age in a curvilinear manner. Intra-cow repeatabilities were (1) 0.61 and (2) 0.56, showing the 305-day record (1) to be slightly more desirable. Heritability estimates calculated from paternal half-sib correlations were 0.93 and 0.97. Doubling the intra-breed dam-daughter regression coefficient obtained from 87 dam-daughter pairs resulted in a heritability estimate of 0.57.

Owing to the decrease in market value of butterfat in recent years, greater emphasis has been placed on the solids-not-fat (SNF) content of milk in the United States and many other countries. This gradual change has focused more attention on the factors which affect SNF content. Although there have been numerous studies on this subject, the majority was based on laboratory procedures which by nature did not lend themselves to the assay of mass data by field techniques. This investigation was undertaken to measure and evaluate the influence of several environmental and genetic factors upon SNF content of milk as determined by procedures applicable to field use.

Over a period of several years, downward trends in SNF content in England were noted by Bailey (7) and by Provan and Jenkins (48); conversely, an increase was indicated in the Netherlands by Janse (30). Little systematic change in either direction was found in England by Baker and Cranfield (8) or in Scotland by Waite *et al.* (60). Long-time trends were often difficult to evaluate because of changes in assay procedures. In nine English herds studied by the

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Milk Marketing Board (38) over a 4- or 5-yr. period, the smallest range in yearly averages was 0.08%, the largest was 0.34%. Both were Holstein herds.

A review of 14 investigations by Herrmann (26) showed changes in average monthly SNF percentages of varying degree. There, and in other studies throughout the world, SNF content was shown to be lowered by high temperatures (14, 28), low nutritive plane (6, 39, 53, 60), and drought (22, 33, 49), or combinations of these factors (11, 12, 18, 23, 25, 29, 32, 35, 37, 38, 48, 57, 58). With optimum conditions, it appeared that SNF content increased. Results of the individual studies varied according to local conditions.

Changes in SNF content during the lactation also were described by Herrmann (26) in a review of nine investigations. In general, percentage dropped from an initial high level shortly after parturition to a lactation low between 1 and 2 mo., remained generally constant for 5 to 6 mo., and gradually increased during the remainder of the lactation. Similar trends have been described by others (5, 15, 18, 25, 38, 46, 55, 57, 60). The effect of month of lactation was found to be statistically significant by Nicholson *et al.* (40) with 18 Holsteins over a 3-yr. period. On more limited data, dissimilar lactation trends were observed (35, 51). To remove the confounding effects of month of year on stage of lactation, Waite *et al.* (60) obtained estimates of each effect independent of the other. The common stage-of-lactation curve still remained.

Investigating the effect of pregnancy, Bartlett (9) and Rowland (54) noted increases between the 4th and 5th mo. of gestation which accompanied the characteristic decline in milk yield. In farrow cows, SNF percentage seemed to decrease slightly as the lactation progressed. Bailey (5) noticed pregnancy effects after the 4th mo. for mature cows and slightly later for first- and second-calf heifers. Tests from 6 wk. to 6 mo. were recommended as being the most indicative of SNF percentage for investigational work. Gorrie and Harvey (23) found stage of pregnancy to be the largest of several factors measured. Effects were apparent after 100 days.

In seven investigations reviewed by Herrmann (26), SNF percentage was shown to decline with age at a rate varying between 0.03 and 0.18% per lactation. Further confirmation was available from Bailey (4), Edwards (18), Flux *et al.* (19), and White and Drakely (64). Gowen (24) noted that decreases due to age were linear, and amounted to 0.024% per year. A decrease of about 0.05% per lactation was noted by Waite *et al.* (60), and 0.10% by Griffiths and Featherstone (25) and the Milk Marketing Board (38).

Relatively few heritability estimates have been reported. Several investigations reviewed by Tyler (59) indicated a positive genetic influence. Most comprehensive of those reviewed were the investigations of Robertson *et al.* (50), who estimated the heritability of SNF content to be 0.53, and Politiek (47), who estimated it to be between 0.5 and 0.7. Shaw and Fourt (55) and the Milk Marketing Board (38) also presented evidence of genetic influence. By doubling the intra-sire daughter-dam regression from 146 pairs of two breeds, Johnson (31) calculated heritability estimates of 0.35 and 0.34. The repeatability of SNF content of consecutive lactation records was reported by Wilcox *et al.* (65)

to be 0.80 when data from 70 cows of two breeds were appropriately combined. Flux *et al.* (19) estimated repeatability of 495 records of 161 cows to be 0.75.

INVESTIGATIONAL PROCEDURE

Determinations on a few selected cows were made as a preliminary step from July through September, 1953. Routine samples were subsequently taken on every cow of the Holstein and Guernsey herds of the New Jersey Agricultural Experiment Station on or about the 15th of each month. By February, 1958, SNF content had been determined in 4,772 samples from 222 cows. Unweighted night and morning samples were combined. Although slight differences in SNF content between night and morning samples have been suggested by several (3, 9, 15, 17, 21, 22, 23), these differences were not always considered important and were not always in the same direction. No significant differences were found by several others (20, 24, 32, 37, 41, 45, 57, 58).

Until June, 1955, SNF percentage was measured by subtracting fat percentage, as determined by the Babcock fat test, from total solids percentage, as determined from A.O.A.C. tables for Quevenne Lactometer readings (2). Subsequently, a Watson Pattern Lactometer (62) was used and SNF percentage calculated by subtracting fat percentage from total solids percentage as determined from USDA tables (61). Comparison of estimates of SNF percentage by the two methods on milk of the New Jersey Agricultural Experiment Station resulted in a correlation of +0.92 (65), with no difference in the mean values. The superiority of the Watson Pattern Lactometer was demonstrated by Robinson *et al.* (52), but not by Cosgrove and Cobble (10) or Madden *et al.* (36).

To avoid possible confounding of month and stage of lactation due to disproportionate subclass numbers, these effects were measured independently of each other by a method shown by Patterson (43) to be comparable to the method of fitting constants on data provided by Snedecor (56).

Milk samples were taken and tested by assistants in conjunction with the regular DHIA supervisor. No samples were taken before the seventh day after freshening. Records were kept in the DHIA book such as could be a standard practice for commercial dairy farms.

Lactation averages were calculated by dividing the total pounds of SNF by the lactation milk yield. Records ranged from 260 to 305 days in length, the minimum being well within the range recommended by Politiek (47). Since tests taken from the 2nd to 6th mo., inclusive, have been shown to be quite consistent (5), because this portion of the lactation curve approximated a horizontal line, an additional average was calculated using the tests in this interval.

To measure the systematic effects of age, and to obtain estimates of repeatability and heritability, analytical procedures described by Snedecor (56) and Anderson and Bancroft (1) were utilized.

RESULTS AND DISCUSSION

Effect of year. Annual means for SNF percentage from samples taken in the years 1954-57 are presented (Table 1). Samples taken in late 1953 and early

TABLE 1
Mean SNF content of samples from four calendar years

	Breed					
	Holsteins			Guernseys		
Year	Cows	Samples	Mean	Cows	Samples	Mean
	(No.)	*(No.)*	*(%)*	*(No.)*	*(No.)*	*(%)*
1954	96	751	8.38*	35	289	8.90
1955	98	792	8.53	34	294	8.90
1956	104	786	8.56	28	290	9.09*
1957	96	787	8.54	37	342	8.91
Over-all	156[a]	3,116	8.50	62[a]	1,215	8.93

* Differs significantly ($P < 0.05$) from other 3 yr.
[a] Not a sum; most cows represented in several years.

1958 have been omitted, since they represented only a portion of these years. The mean values represented averages of sample percentages only. Limiting this phase of the investigation to four calendar years caused the exclusion of all data from three Holsteins and one Guernsey, as well as a number of samples from other cows; thus, 4,331 samples from 218 cows were included.

An analysis of variance of the unweighted monthly means showed significant differences among years. A major portion of this variation was due to the high year for Guernseys (1956) and to the low year for Holsteins (1954). Applied on a within-breed basis, the Duncan Multiple Range Test (16) indicated that only these years differed from the remaining three. A factor in the high 1956 Guernsey average was an exceptionally high January test (9.64%). On this test day fat percentage (5.2%) for Guernseys was also the highest for the 4-yr. period; at the same time, a 4-yr. high (8.81%) was established for Holstein SNF percentage. The low year for Holsteins (1954) seemed due to low tests in June and July. A portion of the variation could have been attributable to changes in the population over the 4 yr. However, since no attempt to select for SNF content was made, since only about 20 to 25% of the cows were replaced each year, and since the replacement daughters could be expected to have about the same inherent level as the existing population of dams, the change in SNF content caused by a change in the make-up of the herd would be expected to be very small. Therefore, the variation among years was considered to be due largely to effects other than animal differences.

No general trends upward or downward were apparent over the short period covered; the odd year was different for each breed and the shift was in an opposite direction. No attempt was made to adjust the data for year effects. It was interesting to note that even though the station Holstein herd tested higher (4.0%) for butterfat than the breed average, there was no accompanying superiority for SNF percentage.

Effect of month and stage of lactation. Analyses of month-of-year and stage-of-lactation effects were carried out with data limited to those samples collected on the first ten monthly sampling days following parturition. The first sample from each cow was taken between seven days post-partum and seven days plus 1 mo. These procedures were purposely followed to make them comparable to

DHIA practices. Of course, similar distributions of the different times at which cows were first sampled would be expected for each month of the year. Furthermore, if changes are similar to those in fat percentage, the major differences associated with this variation would be in the first month, and with a uniform distribution throughout the month, the average can be considered as representative for comparison with later samples. After excluding samples taken after the tenth sampling period, 3,139 Holstein and 1,223 Guernsey samples remained.

Several analyses of variance were carried out on each breed. The basic data were analyzed to point out the major effects, and to establish the within-subclass (error) variance estimate. As a comparative method, an analysis of variance of the unweighted means of the month by stage-of-lactation subclasses was completed. Finally, the data were analyzed after adjustment of each main effect for the other. The sources of variation, significance of mean squares, and estimates of variance and variance percentage are presented (Table 2) for the latter two analyses.

Owing to the removal of the confounding of month and stage of lactation and the nature of the analyses, the analyses of the adjusted data presented the best estimates of the factors measured. Similar significance and estimates of variance and variance percentages resulted from all three analyses. The method of unweighted means has been considered appropriate for preliminary analysis and usually adequate where disproportion of subclass numbers was not great. Because of simplicity, the method has much to recommend it. Although the subclass values ranged in number from 1 to 28 with Guernseys and from 3 to 54 with Holsteins, the added accuracy of the exact method did not seem to justify the extra effort required.

It was obvious that both month and stage of lactation independent of each other were real effects of appreciable size. The variance estimate for months was greater in the Holstein data than in the Guernsey data, but not significantly so. The variance of stage of lactation was significantly greater for Holsteins than for Guernseys. Thus, percentage-wise, the portion of the total variance attributable to months was smaller with Holsteins than Guernseys. Breed differences were noted by Davis *et al.* (13), who found that the range of serum solids percentage during the lactation was largest with Guernseys (1.08%), less with Holsteins (0.65%), and least with Jerseys (0.35%). Stage of lactation seemed to have more effect on Holsteins than Shorthorns in a study of several herds of each breed by the Milk Marketing Board (38).

The actual adjustment factors used to remove the confounding of month and stage-of-lactation effects were small. The largest factor amounted to only 0.08%; the majority were 0.02% or less. In light of the degree of accuracy of the SNF determinations, the adjustment process may not be necessary, or even justified.

The interaction between month and stage, though highly significant, accounted for a very small portion of the variance. The data were graphed, but yielded little further information of any systematic trends, such as a greater month-of year effect in early lactation than in later lactation.

TABLE 2

Variance and variance percentages for month and stage-of-lactation effects as determined by two methods of analysis

Source of variation	Holsteins					Guernseys				
	d.f.	Unweighted means		Adjusted data		d.f.	Unweighted means		Adjusted data	
		(V.)	*(%)*	*(V.)*	*(%)*		*(V.)*	*(%)*	*(V.)*	*(%)*
Month	11	0.244**	30	0.203**	27	11	0.126**	41	0.114**	41
Stage of lactation	9	0.367**	46	0.353**	48	9	0.044**	14	0.035**	13
Interaction	99	0.016**	2	0.011**	1	99	0.020**	7	0.009**	3
Residual	3,019	0.174	22	0.174	23	1,103	0.118	38	0.118	43

** Significant at the 1% level.

Error variation seemed greater among Holstein SNF estimates; comparison of the residual terms showed the difference to be highly significant. It must be pointed out, however, that with 1,103 and 3,019 degrees of freedom, even very small differences can be highly significant. The residual was composed of various random effects of which the variation among animals would seem to be the largest (65). It was assumed in this investigation, as in each of the investigations previously reviewed, that differences among animals would not be confounded with stage-of-lactation and month-of-year effects in such a way as to introduce any important bias. Such a bias would have been possible had cows with unusually high or low SNF percentages happened to fall in subclasses with very small numbers, but subclasses with small numbers did not seem to be out of line with subclasses with larger numbers. Furthermore, since the average calving interval in this herd was about 13 mo., many cows in a given stage of lactation would not be represented in the same month of the year in successive lactations.

Graphic portrayals of month (Figure 1) and stage-of-lactation (Figure 2) effects are presented. Month effects were generally similar for both breeds, with SNF content moving in the same direction at the same time. An exception was the change from August to September, which was downward for Guernseys and slightly upward for Holsteins. Depending on local environmental conditions, various investigators have reported the most important causes of month effects to be temperature, feed, or drought. With this population, rainfall was adequate during the period investigated; a combination of changes in temperature and feed was probably responsible for the month effects.

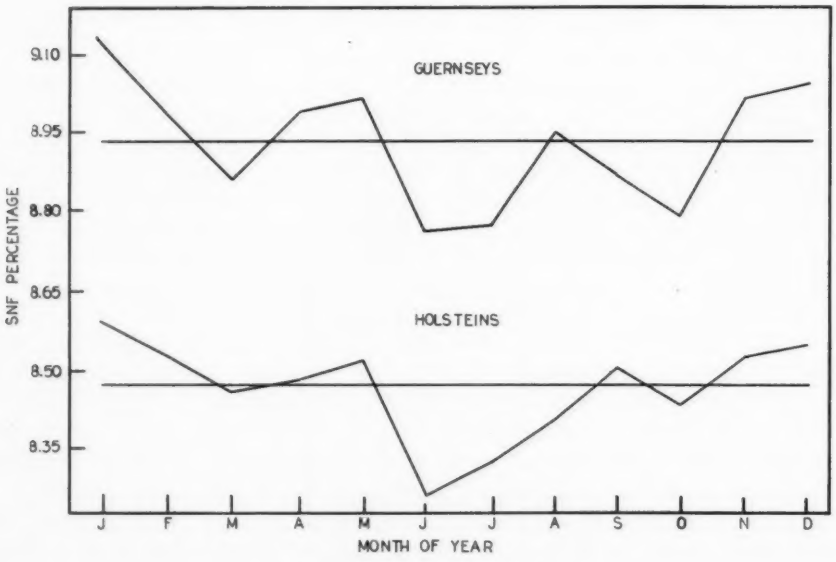

FIG. 1. Effect of month on SNF content.

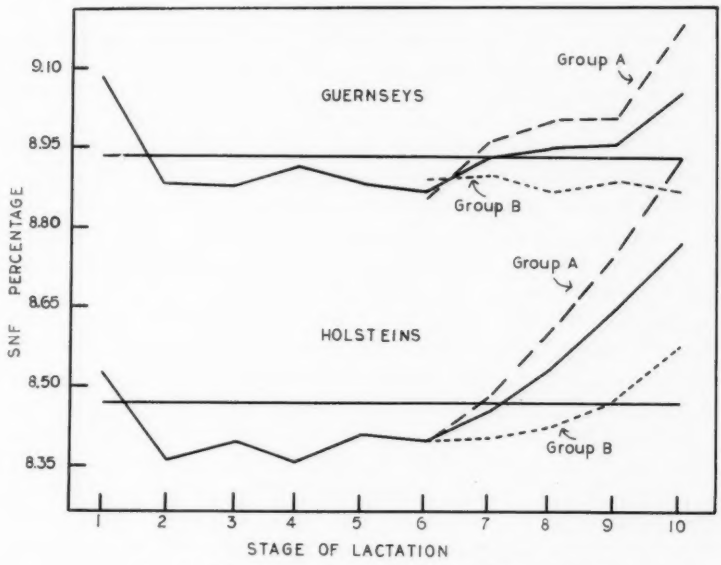

FIG. 2. Effect of stage of lactation on SNF content.

The stage-of-lactation curve was similar to those presented in the literature. The initial high value, the steep decline, leveling off, and gradual climb seemed to be independent of breed, location, and investigator.

Effect of gestation. Samples taken from the sixth through tenth test period of each lactation were divided into two groups for each breed. Group A consisted of samples from those animals successfully bred between 50 and 118 days postpartum, thus freshening in less than a 400-day calving interval. Cows conceiving later, or not at all, were placed in Group B. The groups were kept intact throughout, and the mean SNF content for the successive tests presented (Figure 2). The original stage-of-lactation lines were thus divided into two components according to the stage of gestation. Assuming that month effects were the same for both groups, and since the estimates for month effects had been determined from the combined data, percentages in both groups were adjusted by use of the same factors.

SNF levels for Group A and Group B animals were not appreciably different at the sixth test period for either breed. Since the average date of conception of Group A animals was probably near the midpoint between 50 and 117 days, the seventh test period corresponded to the 4th or 5th mo. of pregnancy and demonstrated a pregnancy effect similar to that found by Bartlett (9), and if all age groups were combined, by Bailey (5).

With the exception of the first milk test, where the effects of parturition contributed to high SNF content, much of the so-called stage-of-lactation effect was due to pregnancy with the Holsteins, and practically all could be so ex-

plained with the Guernseys. Further, since the make-up of Group A of each breed was similar in regard to stage of gestation, the greater stage-of-lactation effect found in the Holstein data could not be so explained. Reproductive efficiency was roughly similar for each breed, since 63% of all Holstein determinations, and 60% of all Guernsey determinations, were placed in Group A.

The Group B samples were further divided according to stage of pregnancy. This additional breakdown has not been shown because the loss of numbers made the lines more erratic and specific animal effects became apparent. Although stage of pregnancy was similar in the additional groups, it was found again that its effect was greater with Holsteins than with Guernseys. Although the differences between breeds could be suspected to be due to different management practices, this was unlikely, since the two breeds were kept at the same location with common housing, the same labor, and under a single herdsman.

Effect of age. The mean SNF content of 310 completed Holstein records was 8.48 and 110 Guernsey records 8.95%. These averages were based on all milk samples taken in the first 305 days of each lactation. Averages based on the second through the sixth milk test (five-test average) were 8.42 and 8.93%, respectively, for the two breeds. Represented in the study were 182 cows by 50 different sires.

Records were not adjusted for environmental effects. Stage-of-lactation and pregnancy effects should have been similar in consecutive lactations; if not, the second measure of average SNF content (based on the second through sixth milk test) should have eliminated their effects. Although month-of-year has been shown to account for 27 to 41% of the variation among individual samples, a lactation record based on a series of tests over an extended period of time need not necessarily be biased by month effects. Such a biasing effect could be described as a month-of-freshening effect. White and Judkins (63) believed earlier that month-of-freshening did affect the lactation average, but recent studies by Bailey (4) and the Milk Marketing Board (38), incorporating considerably more data and more sensitive statistical analyses, have indicated otherwise. Adjustments for month-of-freshening accounted for only 1% of the total variation in a study by Robertson *et al.* (50), who indicated that the mentioned adjustment was definitely not justified. On the basis of these previous reports, and the erratic nature of the month-of-year effects, records have not been adjusted for month-of-freshening for this investigation.

Records were arranged according to lactation number for both breeds and for both methods of calculation (Table 3). A gradual, though irregular, decrease of SNF content with age was noted. Preliminary study of the averages showed a departure from linearity, the cubic term being significant. Therefore, the cubic curve was calculated on an among-record-within-cow basis for each breed and for the 305-day and five-test records. The genetic differences among animals were, thus, not involved in the calculations. The constants were all significant or highly significant (Table 4), supporting the preliminary calculations. In the order listed in the table, the combined effects of the regressions represented 13, 12, 25, and 24% of the within-cow variation among SNF records.

TABLE 3

Effect of age on SNF lactation average

Lactation number	Holsteins			Guernseys		
	Records	305-day	5-test	Records	305-day	5-test
	(No.)	*(%)*	*(%)*	*(No.)*	*(%)*	*(%)*
1	80	8.68	8.63	28	9.18	9.16
2	67	8.55	8.49	18	8.96	8.92
3	51	8.43	8.35	16	8.83	8.80
4	40	8.35	8.28	16	8.89	8.84
5	28	8.38	8.31	14	8.86	8.84
6	17	8.25	8.17	9	8.85	8.86
7-10	27	8.23	8.18	9	8.85	8.80
Total or av.	310	8.48	8.42	110	8.95	8.93

TABLE 4

Constants representing the effects of age on SNF content

Type of record	Intercept	Partial regression		
		Linear	Quadratic	Cubic
Holstein				
305-day	8.72	−0.1661**	+0.0287**	−0.0015*
5-test	8.68	−0.1853**	+0.0327**	−0.0018**
Guernsey				
305-day	9.43	−0.3714**	+0.0768**	−0.0049**
5-test	9.47	−0.4237**	+0.0883**	−0.0057**

** Significant at the 1% level.
* Significant at the 5% level.

The gradual lowering of SNF content with age was in agreement with other reported investigations. None of those reviewed, however, presented conclusions of curvilinear effects. Whether the decrease was a feature of age per se, or the result of gradual deterioration of udder health or function, has not been studied. Mastitis has been shown to lower SNF content. As cows age, it may be assumed that mastitis attacks occur more frequently, each time causing some permanent damage. Some, if not all, of the so-called age effect could have been caused by progressive udder deterioration.

Repeatability and heritability. Expected SNF values for each lactation number, according to the calculated equations, were determined, and deviations from the Lactation 3 value used for adjusting all individual records. Lactation 3 was used as the adjustment base, since the mean of the lactation numbers was 3.20 and a whole number was considered desirable. Adjusted data were then analyzed separately by breed, and then combined, to give repeatability and heritability estimates. These estimates were obtained through analyses of variance, and portions of the analyses are presented (Table 5).

Repeatability, $C/(C+R)$, amounted to 0.61 and 0.56 for the 305-day and five-test records, respectively. Utilizing z transformation and correction for bias, the 95% confidence limits of these intraclass correlation coefficients were 0.52 and 0.69, and 0.46 and 0.65, respectively. Slightly more within-cow variability existed among the five-test records, but this difference was not significant.

TABLE 5

Analysis of variance of SNF records adjusted for lactation number

Source of variation	Degrees of freedom	Mean squares	Expectation of mean squares	Components of variance
		(305-day av.)		
Sires in breed (S)	48	0.266**	R + 2.389 C + 8.678 S	S = 0.018
Cows in sire (C)	134	0.105**	R + 2.257 C	C = 0.036
Records in cow (R)	236	0.023	R	R = 0.023
Total	418			
		(5-test av.)		
Sires in breed (S)	48	0.296**	R + 2.389 C + 8.678 S	S = 0.021
Cows in sire (C)	134	0.111**	R + 2.257 C	C = 0.036
Records in cow (R)	236	0.028	R	R = 0.028
Total	418			

** Significant at the 1% level.

It had been suggested (5) that the five-test records might be superior. The demonstration of parturition and pregnancy effects in this study indicated that the methods warranted comparison. If repeatability and within-cow variability were to be used as criteria, however, the 305-day record appeared to be the most desirable.

The rather high repeatability indicated that the characteristic was fixed to a considerable degree in an individual. As expected, the repeatability estimate obtained in this study, representing the intra-cow correlation of any record with any other record, was somewhat lower than an earlier reported estimate (65) of 0.80, where the intra-cow correlation of consecutive records was measured.

There seemed to be no advantage in using records based on the second through sixth milk test. Two factors appeared to be the most important causes of the lowered repeatability. First, with the nine or ten samples taken for the 305-day record, more of the random errors due to sampling and testing, etc. would balance than with five samples. Secondly, the effects of season, shown to be large and irregular through the year, would have more opportunity to equalize in the longer records. Both of these factors are important, and both might have acted to the detriment of the accuracy of the five-test average. Since the season effects have not been consistent among the various studies, it is of questionable value to attempt to adjust SNF records specifically for season.

Heritability estimates, $4S/(S + C + R)$, were high (0.93 and 0.97, respectively). The 95% confidence limits of the two correlations obtained (0.23** and 0.24**) were estimated to be 0.03 and 0.43, and 0.04 and 0.44. The problems involved in estimating the heritability of lactation SNF content were not of a simple nature. The limitations in numbers prevented more sensitive analyses. This problem was met with some success in studies of beef cattle by Patterson *et al.* (44), who felt that an illogically high heritability estimate from the intraclass correlation of paternal half-sibs could be attributed to nonisolated interactions. These investigators obtained estimates of several interactions from portions of the data which lent themselves to analysis; on this basis, the effects of major interactions were removed. Further, it was shown that the average relationship of half-sibs was closer to 0.33 than to 0.25, which led to a factor of

3, rather than 4, in the formula for estimating heritability from intra-sire half-sib correlations. Thus, it may be concluded that determinations of heritability are not too satisfactory by the half-sib method when the sample size is small.

From 87 dam-daughter pairs, an intra-breed regression coefficient of 0.28**, with 95% confidence limits of 0.04 and 0.53, was determined. Heritability measured on this basis amounted to 0.57. A number of sires were represented by only one daughter-dam pair, preventing the analysis on a within-sire basis because of the drastic reduction in degrees of freedom. Since all records were more or less contemporary, however, bias due to environmental correlation which would have been removed by an intra-sire scheme of analysis was undoubtedly small.

Lush (34) has indicated that the regression of daughters on dams is the appropriate basis for calculating heritabilities. The regression is used in preference to the correlation coefficient, primarily because of the undesirable effect of selection of the independent variable on the correlation coefficient, in this case the selection of dams. However, the correlation between daughter and dam may sometimes be justified if there has been no selection of dams for the measure being studied, as in this case. The interclass dam-daughter correlation was 0.34**, with confidence limits of 0.13 and 0.52. Doubling these values gave a heritability estimate of 0.68, with a possible range of 0.26 to 1.00.

Assuming, as stated by Lush (34), that repeatability can be used as an estimate of the upper limit of heritability, then some credence could be placed upon the heritability estimate (0.57) obtained from the dam-daughter regression. The estimates of 0.53 found by Robertson *et al.* (50) and of 0.5 to 0.7 found by Politiek (47) would then be substantiated.

CONCLUSIONS

Use of field techniques and indirect SNF determination procedures was adequate to measure the environmental factors included in this investigation. Month, stage of lactation, and pregnancy were shown to influence SNF content of individual samples significantly. Lactation SNF content decreased systematically with age in a curvilinear manner. Averages based on all tests during the first 305 days of lactation were only slightly more reliable than averages based on the 2nd through 6th mo. of lactation. Repeatability and heritability estimates for SNF lactation average were found to be higher than most commonly accepted values for lactation milk and fat yield.

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DISTRIBUTION AND INHERITANCE OF HEMOGLOBIN VARIANTS IN AMERICAN CATTLE[1]

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SUMMARY

The relative frequencies and genetic determination of the two electrophoretically distinguishable adult bovine hemoglobin types were investigated in a large sample drawn from a number of different breeds and sources. Electrophoretic studies of hemoglobins from 121 males and 646 females, representing nine cattle breeds, indicate that the Ayrshire, Holstein, Angus, Hereford, and Milking Shorthorn breeds probably have only Type A hemoglobin; whereas, the Brown Swiss, Guernsey, Jersey, and Sindhi breeds have both Type A and Type B. An analysis of hemoglobin types among progeny from parents of known types substantiates the theory that the occurrence of these two types of hemoglobin is genetically determined by a pair of codominant alleles—HbA, determining Type A, and HbB, determining Type B. Estimates of the frequency of HbB among the major dairy breeds are: Ayrshire—0.000, Holstein—0.000, Guernsey—0.116, Brown Swiss—0.117, Jersey—0.333. No gross physiological differences have been observed to be correlated with differences in hemoglobin type. The trait may be of some practical value in parentage determination and in the tracing of breed origins. The most significant feature of these results is that they represent a further demonstration of single-gene determination of a protein specificity.

Electrophoretically distinguishable hemoglobins have been observed in the blood of cattle of North Africa by Cabannes and Serain (3), of Great Britain by Bangham (1), and of the United States by Grimes, Duncan, and Lassiter (6) and by Salisbury and Shreffler (12). In each instance, two types of hemoglobin have been observed which migrate at different rates in an electrical field. Three studies (1, 6, 12) indicated probable breed differences in the relative frequencies of the two hemoglobin variants, suggesting genetic determination of the trait. In the most extensive study reported thus far, Bangham (1) presented data indicating that these two types of hemoglobin are genetically controlled by a pair of alleles at a single locus, with neither allele exhibiting any apparent degree of dominance, so that in a heterozygous individual the blood contains a mixture of the two types of hemoglobin. Salisbury and Shreffler (12) reported preliminary observations which seemed to support this theory. The present paper reports the results of a more extensive survey of cattle of a number of breeds and from a number of sources. The objective of this survey was to obtain further evidence establishing the mode of inheritance of these hemoglobin types and to obtain estimates of the gene frequencies and genotypic distributions within breeds.

EXPERIMENTAL PROCEDURE

Animals. Included in this study were 121 males and 646 females, representing nine breeds and drawn from four experiment station herds and the two

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Illinois artificial breeding centers. The breeds represented were the five major American dairy breeds plus the Sindhi, Milking Shorthorn, Angus, and Hereford breeds. The latter three breeds were represented only by bulls from the artificial breeding centers. The Holsteins, Guernseys, and Ayrshires studied were all in the University of Illinois herd, except 42 Holstein bulls and 21 Guernsey bulls, which were sampled at the two artificial breeding centers. Brown Swiss were sampled from the University of Illinois herd, the artificial breeding centers, the North Louisiana Hill Farm Experiment Station at Homer, Louisiana, and the USDA herd at Beltsville, Maryland. Jersey bloods were obtained from the same sources as the Brown Swiss, plus the Iberia Livestock Dairy Station at Jeanerette, Louisiana. The Sindhis and Sindhi crosses were obtained from the Beltsville, Homer, and Jeanerette stations.

Because previous observations (1, 6, 12) have indicated that the occurrence of fetal hemoglobin, Type F, in very young individuals may be confused with the adult Type B hemoglobin, no animals were included in this survey which were not at least 3 mo. of age. The nature of the similarities and differences between bovine hemoglobin Types B and F has been studied in detail and reported by Grimes, Duncan, and Lassiter (7).

Hemoglobin analysis. A heparinized sample of venous blood was drawn from each animal to be tested. Seven milliliters of 0.9% NaCl solution were added to a 1-ml. sample of the blood, the sample was centrifuged to sediment the erythrocytes, and the supernatant was removed. The erythrocytes were washed twice more by addition of 0.9% NaCl solution, centrifugation, and removal of the supernatant. The washed cells were then hemolyzed with a quantity of distilled water sufficient to give a hemoglobin solution of approximately 10% concentration. The prepared solutions were used either fresh or after storage at 0° F.

For analysis by electrophoresis, 0.01 ml. of the prepared hemoglobin solution was banded upon a strip of Whatman 3 MM filter paper on a Durrum hanging strip electrophoresis apparatus (2) (manufactured by Spinco). Separations were carried out at room temperature for 3 hr. in veronal buffer, pH 8.6, ionic strength 0.05, at 8 v. per cm. In the initial phases of the survey, the strips were dyed in bromphenol blue solution for 6 hr., washed in 5% acetic acid, and fixed in an acetate buffer. However, in the course of the survey, it was established that with the concentrated hemoglobin solution described, the rate of migration could be accurately determined without dyeing, by examination of the visibly colored bands of hemoglobin. Therefore, for the qualitative purposes of this survey, the dyeing was discontinued.

Recent observations indicate also that, for survey work of this type, separation of the hemoglobins from the plasma proteins is unnecessary. Consistently accurate results were obtained by diluting 1 vol. of whole blood with 3 vol. of distilled water, to hemolyze the erythrocytes, and analyzing the resulting solution by electrophoresis. The strips can not be dyed, but the position of the hemoglobin can be determined by its characteristic color, as previously discussed. No results were obtained which indicated interference in normal migration of the hemoglobins due to the presence of the plasma proteins.

RESULTS

Three specific types of results were obtained in the analysis by paper electrophoresis. One type of hemoglobin, designated Type A, migrated a mean distance of 1.5 cm. under the experimental conditions described. Another type, designated Type B, migrated a mean distance of 2.5 cm. under these conditions. A third type of result apparently arose from the presence of both Type A and Type B hemoglobins in the blood of some individuals. This result, called Type AB, was characterized by two bands of hemoglobin, one migrating 1.5 cm., the other migrating 2.5 cm. A mixture of equal quantities of blood containing only Type A hemoglobin and blood containing only Type B hemoglobin was found to give a similar result. The specific hemoglobin type of any individual is entirely repeatable, both for duplicate analyses of the same sample of blood and for analyses of samples taken at different times. Re-analysis of 35 adult individuals after intervals of a year or more revealed no instance of change in hemoglobin type.

Table 1 shows the distributions within breeds of hemoglobin types among the unselected animals analyzed. Those individuals tested as a part of the genetic study because of their presumptive or predicted hemoglobin type are excluded from these data, and only those individuals sampled at random are included. Type A hemoglobin was found among all of the breeds studied, and was found to be the sole type among the Holstein, Ayrshire, Milking Shorthorn, Angus, and Hereford individuals tested. Types B and AB were observed only in the Brown Swiss, Guernsey, Jersey, and Sindhi breeds. The 42 Ayrshires tested provide rather sound evidence that if Type B hemoglobin occurs in this breed, its frequency must be quite low. This is supported by the observations of Bangham (1), who found only Type A among 38 Ayrshire bulls, and of Grimes, Duncan, and Lassiter (6), who found only Type A among eight individuals. The absence of Type B among the large number of Holsteins tested suggests that this trait occurs very rarely, if at all, within this breed. This is also in agreement with the results of Bangham (1) and Grimes, Duncan, and Lassiter (6) from smaller numbers of animals. The numbers of Shorthorn, Angus, and Hereford bulls are much too small to suggest any hemoglobin characterization of these breeds, but the results, which show the presence of Type A only, are consistent with those of Bangham (1), which included larger numbers of individuals.

The size and the variety of sources of the sample of Brown Swiss should make it possible to characterize this breed reasonably well with respect to this trait. Approximately one-fourth of the Brown Swiss have Type AB hemoglobin, while about 1% are of Type B. The remainder are Type A. Although the sample is smaller, and probably less representative, the results for the Guernseys sampled indicate a frequency of hemoglobin types similar to that found for the Brown Swiss. The sample for the Jersey breed, drawn from five different sources, should also be reasonably representative of the breed. The analysis of this sample indicates a higher frequency of Types B and AB than found for the Guernsey

TABLE 1

Distribution of hemoglobin types within breeds

Breed	Sex	Total	Hemoglobin type		
			A	AB	B
Ayrshire	F	42	42	0	0
Brown Swiss	F	120	91	28	1
	M	17	15	2	0
		137	106	30	1
Guernsey	F	51	42	9	0
	M	22	15	6	1
		73	57	15	1
Holstein	F	112	112	0	0
	M	43	43	0	0
		155	155	0	0
Jersey	F	77	41	25	11
	M	13	3	7	3
		90	44	32	14
Sindhi	F	1	0	0	1
	M	1	0	0	1
		2	0	0	2
Milking Shorthorn	M	12	12	0	0
Aberdeen-Angus	M	9	9	0	0
Hereford	M	5	5	0	0
Crossbred					
Holstein-Guernsey, F_1	F	45	40	5	0
Holstein-Guernsey, other	F	46	46	0	0
Brown Swiss-Sindhi, F_1	F	14	0	14	0
Brown Swiss-Sindhi, other	F	46	23	21	2
Jersey-Sindhi, F_1	F	9	2	5	2
Jersey-Sindhi, other	F	37	14	17	6
Holstein-Sindhi, F_1	F	2	0	2	0
Holstein-Sindhi, other	F	9	4	5	0

and Brown Swiss breeds. Over one-half of the individuals tested were either of Type B or Type AB.

Although only two pure Sindhis were tested, both of which were Type B, some further indication of frequency of Type B hemoglobin in this breed may be obtained from the data on crosses of the Sindhi with the Brown Swiss, Jersey, and Holstein breeds. These results suggest that Type B hemoglobin is the predominant type in the Sindhi breed, and perhaps the only type, since the frequency of Type B among the F_1 crosses of Sindhi with European breeds is higher than that found in any of the European breeds involved in the crosses. Examination of data on individual crosses in which the hemoglobin type of the European dams is known reveals that in all but one instance the Sindhi sires used in the crosses were Type B, assuming that the proposed theory of inheritance of the characteristic is correct. It must be pointed out, however, that

most of the Sindhi individuals tested, or involved in the crosses the progeny of which were tested, were rather closely related. Thus, these observations may reflect the hemoglobin distribution in only a particular family or line of the breed, rather than the breed in general.

In connection with the two-allele theory of inheritance of the trait suggested by Bangham (1), the authors have proposed (12) that the genes controlling these hemoglobin types be designated as HbA, controlling the less rapidly migrating Type A, and HbB, controlling the more rapidly migrating Type B. Thus, according to this nomenclature and the suggested mode of inheritance, an individual HbA/HbA would have only Type A hemoglobin, an individual HbB/HbB would have only Type B, and a heterozygous individual, HbA/HbB, would have both Type A and Type B, the condition which has been designated as Type AB.

Table 2 contains the distributions of hemoglobin types among the offspring resulting from matings of sires and dams of known types. Included are the actual distributions observed among the offspring in each class of matings, and the distributions expected with genetic control by a pair of codominant alleles. Since the presumptive heterozygous condition occurs both in males and in females, and since the ratios among offspring of reciprocal crosses show no significant differences, the possibility of sex linkage is effectively eliminated. Therefore, in the data presented in Table 2, reciprocal crosses have been pooled. Chi-square and probability values for the A × AB crosses indicate how well the observed distribution fits the expected. The numbers of animals in the AB × AB and B × AB classes are too small for any meaningful test of fit, but it will be noted that the observed and expected distributions are quite comparable in these classes. It should also be observed that no offspring was produced in any of these crosses which was not possible under the two-allele theory of inheritance. This is of particular importance in the critical tests, A × A, B × B, and A × B, in which all of the progeny of a given mating class must have identical genotypes. Larger numbers among the crosses involving Types B and AB would, of course, be desirable. The numbers are limited because the low frequency of individuals with these types among the breeds from which most of the data were drawn makes such matings infrequent.

In Table 3 may be found similar data for a number of instances in which the hemoglobin type of only one parent was known. While the small number of animals in the AB and B groups, and the division of these animals among several breeds, makes a test for the fit of the ratios of questionable reliability, values are included for a Chi-square test. The distribution of genotypes among the unknown parents was estimated from the breed gene frequencies in Table 4. Expected distributions of progeny were calculated on this basis. The Chi-square and probability values for these groups indicate that the observed distributions do not show deviations from the expected distributions which can not be attributed to chance. Also, it should again be observed that no offspring occurred among these groups which could not possibly occur under the proposed mode of inheritance.

TABLE 2

Distribution of hemoglobin types among offspring of parents of known type

Parental types	Value	Total	Hemoglobin type			χ^2	Probability
			A	AB	B		
A × A	Obs.	78	78	0	0		
(HbA/HbA × HbA/HbA)[a]	Exp.	78	78	0	0	.....	.....
A × AB	Obs.	45	27	18	0		
(HbA/HbA × HbA/HbB)	Exp.	46	23	23	0	1.778	0.20>P>0.10
AB × AB	Obs.	3	1	2	0		
(HbA/HbB × HbA/HbB)	Exp.	4	1	2	1	.....	.....
A × B	Obs.	4	0	4	0		
(HbA/HbA × HbB/HbB)	Exp.	4	0	4	0	.....	.....
B × AB	Obs.	5	0	3	2		
(HbB/HbB × HbA/HbB)	Exp.	6	0	3	3	.....	.....
B × B	Obs.	5	0	0	5		
(HbB/HbB × HbB/HbB)	Exp.	5	0	0	5	.....	.....

[a] Presumptive genotype.

If the theory of inheritance is correct, the phenotypic distributions of hemoglobin types shown in Table 1 are also genotypic distributions, and the frequencies of the genes controlling these types may be calculated from these data. Because there were apparent differences in the frequencies and distributions between males and females within breeds having both hemoglobin types, the distributions of males and females within these breeds were first tested separately by the Chi-square test for homogeneity of a binomial distribution (13). This test showed the separate distributions of Brown Swiss, Guernsey, and Jersey males and females to be homogeneous with respect to their own frequencies, although the probability that the deviations in Jersey females were due to chance was very close to the 0.05 level. The differences in frequencies between males and females within breeds were then tested (13). It was found that none of these differences was significant. Therefore, gene frequencies and the test for equilibrium of the breed populations were calculated for the pooled samples of males and females within breeds. The gene frequencies were calculated in the usual manner for a two-allele trait without dominance (10), and are presented in Table 4. If random mating is assumed, the expected genotypic array may be calculated from these frequencies and compared with the observed genotypic distribution in the usual test for equilibrium of a population (10). The Chi-square and probability values given in Table 4 for the three breeds in which both hemoglobin types occur indicate no significant departure in the observed frequencies from those expected with random mating in a two-allele system.

DISCUSSION

The evidence in Tables 2 and 3 is consistent with that obtained by Bangham (1), upon which the theory of genetic control was originally based, and serves to support and strengthen that hypothesis. This mode of inheritance is also consistent with findings with respect to hemoglobin variants in humans (9), sheep (4), and mice (5), although codominance in the genetic determination of mouse hemoglobins has not yet been demonstrated.

TABLE 3

Distribution of hemoglobin types among offspring of one parent of known type

Parental type	Value	Total	Hemoglobin type			χ^2	Probability
			A	AB	B		
A × ?	Obs.	116	97	19	0		
(HbA/HbA)[a]	Exp.	116	101	15	0	1.225	0.30>P>0.20
AB × ?	Obs.	25	6	13	6		
(HbA/HbB)	Exp.	26	9	13	4	2.000	0.50>P>0.30
B × ?	Obs.	9	0	5	4		
(HbB/HbB)	Exp.	9	0	5	4	0.000	P=1.00

[a] Presumptive genotype.

While it can not be considered as direct or definite evidence, the hypothesis is also supported by the fact that the tests of breed populations imply equilibrium conditions under this type of genetic control. It must, of course, be recognized that certain factors, such as nonrandom mating, selection, and lethal effects, might influence this test for equilibrium. Three explanations may be suggested for the positive test for equilibrium: (1) The theory of inheritance is correct and, with respect to this trait, mating is relatively random with no important lethality or selection. (2) The theory of inheritance is correct, but the effects of lethality, selection, and nonrandom mating act in different directions, in such a way that by chance the test for equilibrium is positive. (3) The theory of inheritance is not correct, but chance combinations of lethal effects, selection, and nonrandom mating have resulted in a phenotypic array which fits the two-gene hypothesis.

Certainly, selection and nonrandom mating are more the rule than the exception in dairy cattle breeding. However, since the trait can be recognized only by the methods previously described, no direct selection based upon hemoglobin type can have been employed in the animals sampled, nor can any departure from random mating have been based upon this trait. Observations in the Illinois station herd have revealed no gross physiological differences within breeds between animals having different types of hemoglobin. Therefore, it does not seem likely that any extensive selection for hemoglobin type due to genetic correlations with other traits has taken place. Also, if either type of hemoglobin caused lethal effects, this would be expected to result in deviations among the ratios of genotypes in the data in Tables 2 and 3. On the basis of these considerations, the first explanation seems the most probable, although the others can not be excluded. It would appear, however, that the evidence presently available dictates the acceptance of the hypothesis of genetic determination by a pair of codominant alleles, until such time as new evidence might disprove it.

It is not presently known whether these observations are of practical significance. These hemoglobin variants could be of some value in parentage determination in certain breeds. Their occurrence and frequency might be useful in tracing breed origins and relationships among certain breeds. Present evidence indicates some common ancestry for the Brown Swiss, Guernsey, Jersey, and Sindhi breeds. Information on the hemoglobin characterization of other Euro-

TABLE 4

Gene frequencies and genetic equilibria within breeds

Breed	q_{HbB}[a]	Value	HbA/HbA	HbA/HbB	HbB/HbB	χ^2	Pobability
Ayrshire	0.000	Obs.	42	0	0		
		Exp.	42	0	0	.....	.....
Brown Swiss	0.117 ± 0.031[b]	Obs.	106	30	1		
		Exp.	107	28	2	0.652	0.50>P>0.30
Guernsey	0.116 ± 0.053[b]	Obs.	57	15	1		
		Exp.	57	15	1	0.000	P=1.00
Holstein	0.000	Obs.	155	0	0		
		Exp.	155	0	0	.....	.....
Jersey	0.333 ± 0.069[b]	Obs.	44	32	14		
		Exp.	40	40	10	3.600	0.10>P>0.05

[a] q_{HbB} = gene frequency of HbB.
[b] ± limits of 95% confidence interval.

pean, African, and Asian breeds could be useful in this respect. As previously stated, no gross within-breed differences in health, vigor, livability, conformation, or production, which could be related to differences in hemoglobin type, have been observed in those animals tested in the University of Illinois herd. It is possible, however, that differences could occur which would be observed only under specific physiological or environmental stresses which "trigger" some physiological failure, as in the case of sickle-cell and other anemias arising from hemoglobin variants in humans (14).

The quantitative investigations of Grimes, Duncan, and Lassiter (6), showing equal concentrations of the two hemoglobin types in what has been shown here to be the heterozygous condition, provide an interesting example of genetic dosage effects. Each allele apparently has equal efficiency with regard to the quantity of hemoglobin produced, but the electrophoretic differences indicate that the molecular structures of the two hemoglobins are slightly different. Thus, these two alleles have quantitatively equal, but qualitatively different, effects. That electrophoretic differences reflect structural differences in the hemoglobin molecule has been conclusively demonstrated by the work of Pauling *et al.* (11), and the later work of Ingram (8). This difference and the simple genetic determination of it are the most significant features of these results. They constitute further evidence supporting the role of genes as determinants of protein specificities.

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ASSOCIATIONS BETWEEN SERVICE INTERVAL, INTERVAL FROM FIRST SERVICE TO CONCEPTION, NUMBER OF SERVICES PER CONCEPTION, AND LEVEL OF BUTTERFAT PRODUCTION

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SUMMARY

The interval from first service to conception decreases as the service interval increases from 0 to 127 days, at which it reaches a minimum. After a service interval of 127 days, the interval from first service to conception increases slightly as the service interval increases. The length of the service interval accounts for only 1.4% of the variance of the interval from first service to conception; thus, even though its effect on the interval from first service to conception is significant, its effect is small and of little importance.

Service interval does not significantly affect the number of services per conception and accounts for only 0.3% of the variance of services per conception. In general, the number of services per conception *(Y)* decreases linearly as the service interval *(X)* increases, the partial regression of Y on X for a constant butterfat production being -0.0034 ± 0.0023.

The partial regressions of the number of services per conception and the interval from first service to conception on butterfat production for a constant service interval are $+0.003 \pm 0.001$ and $+0.091 \pm 0.046$, respectively, and are significant at the 0.05 level of probability. The partial regression of the number of services per conception on butterfat production for a constant service interval and constant interval from first service to conception is -0.00004 ± 0.0007 and is not significant. It is concluded that there is no real biological relationship between services per conception and level of butterfat production.

The service interval alone accounts for 16.8% of the variance of the interval from calving to conception. The interval from calving to conception increases at an increasing rate as service interval increases to approximately 50 days. After a service interval of 50 days, the interval from calving to conception increases almost linearly and in an approximate 1 to 1 ratio as the service interval increases.

Calving interval has approximately the same relationship to service interval as has the interval from first service to conception. With the present data, the average service interval must be from 36 to 49 days to maintain an average calving interval of approximately 365 days. If the conception rate was increased to 60%, the average service interval allowed could be increased approximately 11.5 days, and a calving interval of approximately 365 days could still be maintained.

The association between the interval from calving to the first service (service interval) and the number of services per conception has been investigated by a number of workers (6, 11, 12, 19, 21, 23, 24, 25). In general, these workers reported that the services required per conception decreased as the service interval increased to 100–120 days, when the number of services per conception reached a minimum. VanDemark and Salisbury (25), recognizing the importance of the length of the calving interval, suggested that cows should first be serviced 60 to 80 days after parturition, so as to require a relatively small number of services per conception and still maintain a calving interval of approximately 12 mo.

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Trimberger (24) recommended that cows should not be serviced until 50 days or more after parturition.

Gaines (13) found a correlation of 0.039 ± .010 between the service period (the interval from calving to conception) and the first month's milk production; thus, it is evident that the first month's milk yield and service period are practically independent. In another study, Gaines and Palfrey (14) found a correlation of 0.053 ± 0.049 between the average length of the first nine calving intervals and the average yield per day over the nine calving intervals of 186 cows. On the basis of this correlation, the authors concluded that calving interval has no effect on yield and that high-yielding cows are not bred so as to freshen less frequently than low-yielding cows. Several workers (1, 3, 18, 20, 22) have reported that higher-yielding cows require more services per conception, whereas others (2, 4, 10, 13) have reported that there is no relationship between level of production and services per conception.

The associations between the service interval and the interval from first service to conception, and between the number of services required per conception and the interval from first service to conception, have not been well established.

The objectives of the present study are to determine the associations between the service interval, the interval from first service to conception, the number of services per conception, and the level of butterfat production; and to demonstrate some of the consequences of these associations.

SOURCE AND DESCRIPTION OF DATA

The data for this study were taken from the Red Danish Milkrace progeny groups at the Danish progeny testing stations during the testing year 1955–56. All heifers were in their first lactations when the data involved in this study were recorded. The observations consist of 2,056 artificial services on 902 first-calf heifers by 51 Red Danish Milkrace sires. The number of daughters per sire ranged from 14 to 20, with a mean of 17.7. Eight hundred fifty-three of the 902 heifers conceived and these heifers received a total of 1,782 services. Some of the 49 heifers returned to the farmers as nonpregnant may have been pregnant as a result of their last services, but no information on pregnancy examinations was available after the heifers left the testing stations.

The variables involved in this study are days from first service to conception *(W)*, number of services per conception *(Y)*, service interval (days from calving to first service) *(X)*, services per cow *(Y′)*, and first lactation fat production in kilograms *(Z)*. The herdsmen at the various testing stations recorded the calving dates and service dates on barn sheets, and the heifers were diagnosed for pregnancy by veterinarians or technicians, who indicated the service at which conception most likely occurred. The herdsmen then recorded the date of pregnancy examination and the service at which pregnancy most likely occurred. The data in this study on days from calving to first service *(X)*, days from first service to conception *(W)*, services per conception *(Y)*, and all services *(Y′)* were taken from the barn sheets. The first lactation butterfat records are those given in (15) for the Red Danish Milkrace sires.

All cows included in this study were first-calf heifers, all were given ample opportunity to conceive, there was no selection for production among the daughters of a sire, and there was no tendency to give high producers more of a chance to conceive than low producers; therefore, the data should provide unbiased estimates of the relationships between days from first service to conception, services per conception, total number of services, service interval, and butterfat production.

ANALYSIS OF DATA AND RESULTS

The service intervals ranged from 44 to 202 days; there were only 11 below 50 days and only 13 above 150 days, of which four were above 160 days. In Table 1, the mean days from first service to conception, the services per conception, and the total number of services are listed according to the length of the service interval.

The means listed under the service intervals from 44–49 days appear to be slightly larger than those listed under the longer intervals, but other than this there seems to be no consistent trend of the means with respect to the length of service interval.

In Table 2, the analyses of variance for days from first service to conception, for services per conception, and for all services are shown. In no case is the variation between ten-day service interval groups significant at as low a probability as 0.05.

In spite of the general lack of significance of the between-group mean squares, one should not conclude that the length of the service interval is not associated with the length of the interval from first service to conception, with services per conception, or with total number of services per cow. The differences associated with the different lengths of service interval could be confounded with test station differences in management and with service sire differences in fertility. Such differences are likely to be small in the data, because a concerted effort is exerted to standardize management practices at the various stations and the field nonreturn rates for the various service sires used were approximately equal. In addition to the possibility of confounded effects, it is possible that the linear or a curvilinear component or both components of the length of service interval could be significant, as was shown by VanDemark and Salisbury (25).

To avoid the possibility of confounded variables and to more precisely determine the associations of the length of service interval (X) with the interval from first service to conception (W), services per conception (Y), total services (Y'), and butterfat production (Z), the within-sire variations were studied. In addition, the square root of the service interval $\sqrt{(X)}$ was included, to help account for a curvilinear effect.

On observing the butterfat records published in (15), it was obvious that there were variations in days in production and age at calving. The age at calving ranged from 658 to 1,341 days, with a mean of 899 days, and the days in milk ranged from 232 to 370 days, with a mean of 304 days. To adjust the individual records for these variations in age at calving and days in milk, the within-sire

TABLE 1

Mean number of days from first service to conception, number of services per conception, and number of services as distributed by ten-day periods of the service interval

		Length of service interval												
		44-49	50-59	60-69	70-79	80-89	90-99	100-109	110-119	120-129	130-139	140-149	150 and up	Totals
Heifers that conceived														
Days from first service to conception	$\overline{W}$	72.1	43.29	41.14	35.77	36.35	30.04	31.07	38.96	37.00	41.54	40.00	12.42	36.08
	N	10	39	127	177	178	134	86	45	23	13	9	12	853
	σ_w	56.09	39.85	48.79	48.89	47.33	42.31	39.59	46.08	41.14	44.71	42.00	24.52	45.78
Services per conception	$\overline{Y}$	2.50	2.15	2.16	2.10	2.13	2.04	1.95	2.20	2.17	1.92	2.00	1.50	2.09
	N	10	39	127	177	178	134	86	45	23	13	9	12	853
	σ_y	1.43	1.22	1.37	1.45	1.47	1.39	1.19	1.39	1.11	0.95	1.12	0.80	1.37
Per cent conceived on first service		30.0	38.5	40.2	49.7	46.1	44.8	47.7	40.0	30.4	46.2	44.4	66.7	44.9
Heifers failing to conceive														
No. services	$\overline{Y''}$	7.00	6.60	5.78	5.75	5.80	5.50	4.33	4.00	5.50	4.67	3.00	4.00	5.53
	N	1	5	9	8	10	4	3	2	2	3	1	1	49
All heifers														
All services	$\overline{Y'}$	2.91	2.66	2.40	2.26	2.32	2.14	2.03	2.28	2.44	2.44	2.10	1.69	2.28
	N	11	44	136	185	188	138	89	47	25	16	10	13	902
	$\sigma_{y'}$	1.92	1.16	1.68	1.61	1.68	1.50	1.26	1.41	1.42	1.46	1.10	1.03	1.58
Per cent conceived on first service		27.2	34.1	37.5	47.6	43.6	43.5	46.1	38.3	28.0	37.5	40.0	61.5	42.5

TABLE 2

Analyses of variance for days from first service to conception, services per conception, and all services

Source	Heifers that conceived				All heifers	
	Days from first service to conception *(W)*		Services per conception *(Y)*		All services *(Y')* per cow	
	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.
Between ten-day service intervals	11	2,996	11	0.91	11	2.46
Within ten-day service intervals	841	2,084	841	1.88	890	2.50

multiple regression of butterfat production (in kilograms) on age at calving *(A)* and days in milk *(D)* was derived and is Equation (1).

$$\hat{Z} = -18.59 + 0.075\,A + 0.510\,D \qquad (1)$$

By using Equation (1), the butterfat records were adjusted to an average length of lactation of 304 days and an average age of calving of 899 days. Since the ranges in age at calving and days in milk were relatively narrow, it was assumed that a linear equation would be sufficient to adjust the butterfat records.

The within-sire correlations involving service interval *(X)*, the square root of service interval $\sqrt{(X)}$, days from first service to conception *(W)*, services per conception *(Y)*, and adjusted butterfat production *(Z)* are listed in Table 3. The correlation between services per conception and days from first service to conception is large (0.859), as would be expected, but it indicates that the two variables are not identical. By holding one of the two constant, the other would retain approximately 26% of its original variance. It is recognized that services per conception *(Y)* and days from first service to conception *(W)* are not normally distributed for a given service interval. However, judging from Table 1 it is apparent that the variances of *(W)* and *(Y)* are approximately equal for the various intervals of *(X)*. Thus, for estimating the regression coefficients the requirements that the errors be noncorrelated and have the same variance are satisfied, but for making the usual tests of significance the requirements that the errors be normally and independently distributed are not satisfied. Consequently, one should consider the standard errors of regression coefficients involving *(Y)* and *(W)* as rough approximations.

TABLE 3

Within-sire correlations between service interval *(X)*, square root of service interval ($\sqrt{X}$), days from first service to conception *(W)*, services per conception *(Y)*, and adjusted butterfat production *(Z)* for those heifers that conceived

	$\sqrt{X}$	*W*	*Y*	*Z*
X	0.952	−0.042	−0.047	0.082
$\sqrt{X}$		−0.073	−0.045	0.045
W			0.859	0.078
Y				0.065

The above correlations are based on 801 degrees of freedom and the 0.05 and 0.01 critical values are 0.069 and 0.091, respectively.

The interval from first service to conception. The multiple regression of the interval from first service to conception *(W)* on the service interval *(X)* and $\sqrt{(X)}$ was derived on a within-sire basis from the correlations, standard deviations, and means given in Tables 3 and 4. The resulting regression equation is Equation (2).

$$\hat{W} = 110.99 + 0.62\ (\pm 0.24)\ X - 13.88\ (\pm 4.49)\ \sqrt{X} \qquad (2)$$
$$R_{W \cdot X\sqrt{X}} = 0.116 \qquad \text{D.F.} = 800$$

Equation (2) is plotted in Figure 1. In Equation (2), the partial regression coeefficints are significant at the 0.01 level of probability. Although the above multiple correlation coefficient ($R_{W \cdot X\sqrt{X}} = 0.116$) is significant at the 0.01 level of probability, X and $\sqrt{X}$ have accounted for only 1.4% of the variance of the interval from first service to conception *(W)*. From Equation (2), it is apparent that only a minor part of the variance of the interval from first service to conception is associated with variation in the service interval. VanDemark and Salisbury (25) found that only 1.0% of the variance of services per conception was associated with variation in the service interval. One would expect this rather close agreement, since the correlation between services per conception and the days from first service to conception as given in Table 3 is 0.859.

TABLE 4

Means and within-sire standard deviations for *(X)*, $(\sqrt{X})$, *(W)*, *(Y)*, and *(Z)*

	X	$\sqrt{X}$	W	Y	Z
Mean	86.7	9.24	36.08	2.09	204.0
σ	21.4	1.14	44.84	1.39	34.7

On observing Figure 1, it is apparent that the average interval from first service to conception decreases at a decreasing rate as the service interval increases from 36 to 127 days. As the service interval increases from 127 to 169 days there is practically no change in the average interval from first service to conception. If the derivative of $\hat{W}$ with respect to X for Equation 2 is set equal to zero, the minimum average interval from first service to conception is found to occur at a service interval of 127 days. As the service interval increases from 36 to 127, 49 to 127, and 64 to 127 days, the average interval from first service to conception decreases 17.1, 11.2, and 6.6 days, respectively. Thus, it is apparent that relatively long average intervals from first service to conception are associated with service intervals below 60 days.

Since the interval from first service to conception and the service interval are both significantly correlated with butterfat production, as shown in Table 3, one should, perhaps, include Z as an independent variable along with X and $\sqrt{X}$ when predicting W. This was done, and the resulting equation is Equation 3.

$$\hat{W} = 88.51 + 0.55(\pm 0.24)\ X - 12.88\ (\pm 4.50)\ \sqrt{X} + 0.09\ (\pm 0.05)\ Z.$$
$$R_{W \cdot ZX\sqrt{X}} = 0.136 \qquad \text{D.F.} = 799 \qquad (3)$$

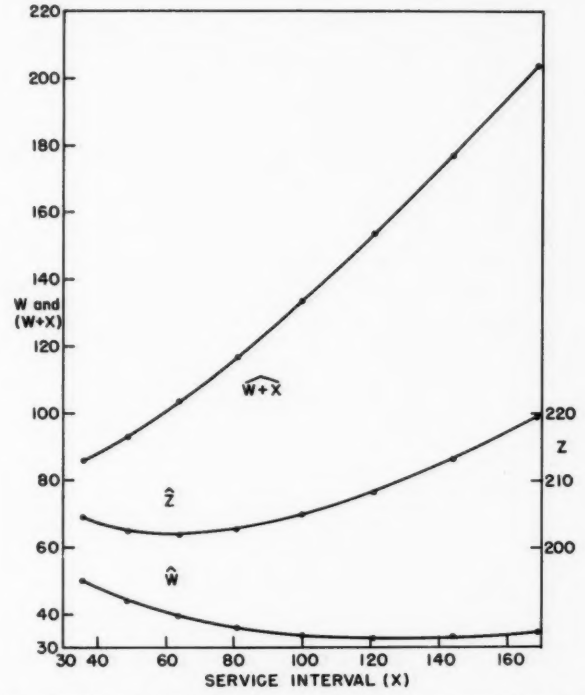

FIG. 1. The regressions of the interval from first service to conception *(W)*, the interval from calving to conception *(W + X)*, and butterfat production *(Z)* on the service interval *(X)*. *Z* is expressed in kilograms and *W*, *X*, and *W + X* are expressed in days.

$$\hat{W} = 110.99 + 0.62\,X - 13.88\,\sqrt{X}.$$

$$\widehat{W+X} = 110.99 + 1.62\,X - 13.88\,\sqrt{X}.$$

$$\hat{Z} = 245.26 + 0.69\,X - 10.90\,\sqrt{X}.$$

If Equation (3) is plotted for a constant *Z* of 204 kg., the resulting graph coincides almost exactly with the graph of Equation (2) in Figure 1. For corresponding regression values to differ more than a day, the service interval must exceed 160 days. Shown in Table 5 are the mean squares of the interval from first service to conception, accounted for independently by the service interval and the level of butterfat production. In general, only a small part of the variance of the interval from first service to conception is associated with variation in the level of butterfat production.

The interval from calving to conception. From the correlations, means, and standard deviations in Tables 3 and 4, the regression of the interval from calving to conception *(W + X)* on the service interval *(X)* and $\sqrt{X}$ can be derived. This regression was derived and is Equation 4.

$$\widehat{W+X} = \hat{W} + X = 110.99 + 1.62\ (\pm 0.24)\ X - 13.88\ (\pm 4.49)\sqrt{X}$$
$$R_{(W+X)\cdot X\sqrt{X}} = 0.41 \qquad \text{D.F.} = 800 \qquad (4)$$

Since service interval is a part of the interval from calving to conception, the regression of $(W + X)$ on X would be expected to be large and the multiple correlation $R_{(W+X)\cdot X\sqrt{X}}$ would be expected to be large.

Equation (4) is plotted in Figure 1. The interval from calving to conception increases markedly as the service interval increases, as would be expected. After a service interval of 49 days, there is an increase in the interval from calving to conception of almost a day for each day the service interval increases. The graph of Equation (4) clearly indicates how prolonging the service interval increases the interval from calving to conception.

The regression of $(W + X)$ on X, $\sqrt{X}$, and Z was derived and is Equation (5)

$$\widehat{W+X} = 88.51 + 1.55\ (\pm 0.24)\,X - 12.88\,(\pm 4.50)\,\sqrt{X} + 0.09\,(\pm 0.05)\,Z$$
$$R_{(W+X)\cdot X\sqrt{X}Z} = 0.42 \qquad \text{D.F.} = 799 \qquad (5)$$

If Equation (5) is graphed for $Z = 204$, the resulting curve coincides almost exactly with that of Equation (4) in Figure 1. The service interval must exceed 160 days for the difference between corresponding regression values to exceed one day.

The level of butterfat production. From the correlations, means, and standard deviations in Tables 3 and 4, the regression of the adjusted butterfat production (in kilograms) on service interval (X) and $\sqrt{X}$ was derived and is Equation (6).

$$\hat{Z} = 245.26 + 0.69\ (\pm 0.18)\ X - 10.90 \pm (3.46)\ \sqrt{X} \qquad (6)$$
$$R_{Z\cdot X\sqrt{X}} = 0.137 \qquad \text{D.F.} = 800$$

Equation (6) is plotted in Figure 1 and, as is evident from Figure 1, the derivative of $\hat{Z}$ with respect to X gives a minimum butterfat production for $X = 63$. As the service interval increases from 63 to 169 days, there is a general increase in the level of butterfat production. As the service interval increases from 36 to 63 days there is a small, but probably not real, decrease in butterfat production. For service intervals from 49 to 90 days there is little difference in the levels of associated butterfat production.

The increase in the level of butterfat production associated with an increase in the length of the service interval could be brought about in several ways: (1) There could be a tendency to breed low-producing cows earlier after calving than high-producing cows. (2) High-producing cows may tend to show estrus at a larger interval postpartum than low-producing cows and, thus, drift into the classes with longer service intervals. (3) With the longer service intervals, conception occurs later after calving; thus, there is less interference of pregnancy with production. The first possible cause is not likely to be of importance, since the management practices were standard for cows at the testing stations. Clapp

TABLE 5

Tests of significance of the within-sire variance of the interval from first service to conception

Source	D.F.	M.S.
Service interval $(R^2_{W\cdot X\sqrt{X}Z} - r^2_{WZ})\ S_W 2$	2	9,991.8**
Butterfat production $(R^2_{W\cdot X\sqrt{X}} - R^2_{W\cdot X\sqrt{X}})S_W 2$	1	7,895.7*
Error $(1 - R^2_{W\cdot X\sqrt{X}Z})\ S_W 2$	799	1,978.8

** Significant at the 0.01 level of probability.
* Significant at the 0.05 level of probability.

(8) has presented information, though not conclusive, that the second cause may be real. Gaines (13), on the other hand, has presented data which indicate that the first full month's milk yield and the service period (the interval from calving to conception) are independent; thus, the second cause is not likely. It is probable that the third possibility is the real cause of the association.

Services per conception. Previous studies (6, 11, 12, 19, 21, 23, 24, 25) have dealt with the number of services per conception in relation to the length of the srvice interval. In the present data, the regression of services per conception on service interval, as shown in Equation (7) and in Table 6, was not significant.

$$\hat{Y} = 1.67 - 0.005\ (\pm 0.007)\ X + 0.03\ (\pm 0.14)\ \sqrt{X} + 0.0028\ (\pm 0.0014)\ Z$$
$$R_{Y\cdot X\sqrt{X}} = 0.084 \qquad \text{D.F.} = 799 \qquad (7)$$

If Equation (7) is plotted for $Z = 204$ kg., the resulting graph shows that there is a decrease in services per conception as the service interval increases; thus agreeing with the results in (6, 11, 12, 19, 21, 23, 24, 25).

Since most of the sum of squares of services per conception associated with service interval was associated with the linear component, the regression of services per conception on the first power of service interval and butterfat production was derived, and the two partial regression coefficients $b_{YX\cdot Z} = -0.0034 \pm 0.0023$ and $b_{YZ\cdot X} = 0.0028 \pm 0.0014$ were found. With 800 degrees of freedom, the partial regression -0.0034 is significant $(0.05 < P < 0.10)$. This result again substantiates previous conclusions that there is a general decrease in the services required per conception as the service interval increases.

TABLE 6

Tests of significance of the within-sire variances of services per conception associated with butterfat production and service interval

Source	D.F.	M.S.
Butterfat production $(R^2_{Y\cdot X\sqrt{X}Z} - R^2_{Y\cdot X\sqrt{X}})\ (S_Y 2)$	1	7.52*
Service interval $(R^2_{Y\cdot X\sqrt{X}Z} - r^2_{YZ})\ (S_Y 2)$	2	2.18
Error $(1 - R^2_{Y\cdot X\sqrt{X}Z})\ (S_Y 2)$	799	1.93

* Significant at the 0.05 level of probability.

TABLE 7

Tests of significance of the within-sire variance of total number of services associated with service interval and butterfat production

Source	D.F.	M.S.
Service interval ($R^2_{Y'\cdot XZ}$) $S_{Y'}2 - r^2_{YZ}S_{Y'}2$	1	12.269*
Butterfat production ($R^2_{Y'\cdot XZ}$) $S_Y2 - r^2_{YX}S_{Y'}2$	1	9.875*
Error ($1 - R^2_{Y\cdot XZ}$) S_Y2	849	2.32

* Significant at the 0.05 level of probability.

The partial regression of services per conception on butterfat production of 0.0028 is significant ($0.01 < P < 0.05$) and suggests that higher-producing cows require more services per conception. This relationship is probably brought about by the fact that cows requiring more services per conception are not pregnant as soon after calving and, thus, that their current production is less affected by pregnancy than is that of cows requiring fewer services per conception.

To provide evidence on this point, the regression of services per conception *(Y)* on service interval *(X)*, the interval from first service to conception *(W)*, and the level of butterfat production *(Z)* was derived and is Equation (8).

$$\hat{Y} = 1.19 - 0.0007 \pm (0.001)\,X + 0.0267\ (\pm 0.0005)\,W - 0.00004\ (\pm 0.0007)Z. \qquad (8)$$

The partial regression $b_{YZ\cdot XW} = -0.00004$ is not significant and is practically zero. Thus, it appears that there is no real biological basis for concluding that high-producing cows are less fertile than low-producing cows. This is in agreement with the conclusions of (2, 4, 10, 13) and in disagreement with the findings of (1, 3, 18, 20, 22). The relationships reported in (1, 3, 18, 20, 22) are, possibly, confounded with other factors.

All services. By studying services per conception, one includes only those cows that conceived. This, in effect, truncates the data to include only fertile cows. Equation (9) is the regression of all services *(Y')*, whether or not the cows conceived, on service interval *(X)* and butterfat production *(Z)*.

$$\hat{Y}' = 2.13 - 0.0056\ (\pm .0024)\,X + 0.0031\ (\pm .0015)\,Z \qquad (9)$$

$$R_{Y'\cdot XZ} = .104 \qquad \text{D.F.} = 849$$

A prediction equation including $\sqrt{X}$ was first derived, but the partial regression of *(Y')* on $\sqrt{X}$ was extremely small and not significant; consequently, it was not included and Equation (9) was used. The regression coefficients in Equation (9) are based on 849 degrees of freedom and are significant at the 0.05 level of probability. The variances independently accounted for by service interval and butterfat production are shown in Table 7. In spite of their significance, the two together account for only 1.1% of the within-sire variance of number of services. For a constant butterfat production, the number of services goes up 0.56 of a service for each 100 days the service interval increases.

DISCUSSION OF RESULTS

The results indicate that the number of services per conception *(Y)* decreases a small, though not significant, amount for each day the service interval *(X)* increases, the equation for a constant level of butterfat production $(\bar{Z})$ being $\hat{Y} = 2.38 - .0034\,X$. The results also indicate that the interval from first service to conception *(W)* decreases at a decreasing rate as the service interval *(X)* increases to 127 days. With a service interval of 127 days, the interval from first service to conception reaches a minimum of 32.8 days.

Both a small number of services per conception and a short interval from first service to conception are important items and are to be desired by a dairyman. However, the attainment of a minimum number of services per conception and a minimum interval from first service to conception are not the only items to be considered in recommending an optimum interval from calving to first service. The interval from calving to conception should definitely be considered, as it and the length of the gestation period determine the length of the calving interval. Most of the variation in calving interval is brought about by variation in the interval from calving to conception, because the variation in the length of the gestation period is relatively small. From results in (9) and Tables 3 and 4 of this study, it appears that about 99% of the variation in calving interval is caused by variation in the interval from calving to conception.

The important thing to remember concerning the service interval, services per conception, the interval from first service to conception, and the interval from calving to conception is: that variation in service interval accounts for approximately 1% of the variation in services per conception and the interval from first service to conception; whereas, variation in the service interval accounts for approximately 17% of the variation in the interval from calving to conception and, thus, of calving interval. Thus, it would seem that the length of the calving interval desired would be the primary consideration in recommending a short or long service interval.

Equation (10) expresses the relationship between the interval from calving to conception and service interval for the mean Z and, as mentioned previously, almost coincides with that plotted in Figure 1.

$$(\hat{W} + X) = 107.13 + 1.55^{**}\,X - 12.88^{**}\,\sqrt{X} \qquad (10)$$

If one assumes that the length of the service interval has little or no effect on the length of the gestation period, then the relationship between service interval and calving interval is expressed by adding the gestation period to Equation (10). Assuming a gestation period of 280 days, the calving interval for a mean Z may be expressed as shown in Equation (11).

$$\text{C.I.} = 387.13 + 1.55\,X - 12.88\,\sqrt{X} \qquad (11)$$

By taking the partial derivative of C.I. in Equation (11) with respect to *(X)*, setting the derivative equal to 0, and solving for X, it is found that the average minimum calving interval occurs when the average service interval *(X)* is

17.2 days. This value of X is outside the range of service intervals in these data and is one that is unrealistic. For service intervals of 36, 49, 64, 81, and 100 days, which are more nearly within the range of the present data, one would expect calving intervals of 366, 373, 383, 397, and 414 days, respectively.

If the expected butterfat production values in Figure 1 for Equation (6) are divided by the corresponding expected calving intervals derived above, the production per calving interval day decreases as the calving interval increases. For example, for service intervals of 36, 49, 64, 81, and 100 days, the expected calving intervals are 366, 373, 383, 397, and 414 days, the expected butterfat production is 205, 203, 202, 203, and 205 kg., and the expected production per calving interval day is 0.56, 0.54, 0.53, 0.51, and 0.50 kg., respectively. The present study agrees with the results of Gaines (14) in regard to current calving interval. Gaines (14) obtained a correlation of -0.134 ± 0.018 between current calving interval and current production per calving interval day, and a correlation of 0.142 ± 0.018 between the previous calving interval and current production per calving interval day. The correlation, 0.142, between previous calving interval and current production per calving interval day would be partially confounded with age effects, since cows having longer previous calving intervals are likely to be older at calving; thus, the correlation of 0.142 is probably too large. Consequently, the conclusion of Gaines (14) that what is gained in the current lactation by having a short calving interval is lost in the next lactation is questionable. Chapman and Casida (7) concluded that there was no marked reduction in production per calving interval day in the second of two consecutive short calving intervals. Johansson and Hansson (17) concluded that the optimum length of the first calving interval would be about 410 to 430 days, and about 400 days for the second. For the third and, probably, later lactations any length between 310 and 430 days gives about the same yield per calving interval day. On a basis of these references, calving intervals of 12 to 13 mo. seem to be desirable.

The real solution for the optimum length of calving interval is, within biological limits, an economic one and depends on the relative economic values of calving intervals of varying lengths, including yield per calving interval day, seasonal variations in the price of milk, number of calves born, and varying number of services required per conception. Under present economic conditions, with the seasonal variations in milk prices, an average calving interval of approximately 12 mo. would seem to be desirable.

It should be remembered that at present under practical conditions man's control over calving interval is limited to that period between the time when the cow first comes into heat after calving and the time when the first service is made. There is more freedom to make this period long than there is to make it short. Artificial breeding organizations would be interested in a low number of services per conception, as this would be less expensive to the organizations where second and third services are given free of charge, when the first or first and second services fail to get the cow in calf. Thus, it is likely that artificial breeding organizations would recommend a relatively long service interval. On the

other hand, a shorter service interval might result in more first services per cow, since the calving intervals would be shorter, thus producing more income for artificial breeding organizations.

If one reduces the number of services required per conception from that found in these data, it would probably be possible to allow a longer service interval and still maintain a 365-day or shorter calving interval. In the present data, the linear regression of the interval from first service to conception *(W)* on services per conception *(Y)* is 27.64; thus, if the average number of services per conception was reduced from 2.09, as found in these data, to 1.67, which would correspond to a 60% conception rate, the interval from first service to conception would be reduced by approximately 11.6 days. Assuming that this effect is the same for service intervals of different lengths, one could allow an average service interval of from 45 to 60 days and still maintain a 365-day calving interval. If one reduced the length of normal gestations by 15 to 30 days, it would be possible to allow a 15- to 30-day longer service interval and still maintain a 365-day calving interval. Although it has not been conclusively established that the gestation period can be shortened without associated deleterious effects, work by DeFries *et al.* (9) indicates that selection for a shorter gestation is feasible.

Other workers (1, 3, 18, 20, 22) have presented data which indicate that high-producing cows require a considerably increased number of services per conception. These data could be biased, in that there would be a tendency to be more patient with high-producing cows and allow them more chance to conceive than would be the case with low-producing cows. It is also possible that service sire differences are confounded with production levels. In addition, it is probable that those cows requiring more services have a longer period from calving to conception and, thus, less effect of pregnancy on lactations.

By utilizing the present data, the partial regression of services per conception on butterfat production for a constant service interval and a constant interval from first service to conception is -0.00004 ± 0.0007. From the correlations, standard deviations, and means in Tables 3 and 4, the partial regressions $b_{r(W+X)\cdot z} = 0.022$ and $b_{rz\cdot(W+X)} = -0.0007$ can be derived. This suggests that the association between the level of butterfat production and services per conception is largely a result of the corresponding relationship between the interval from calving to conception and butterfat production; thus, there is no evidence in these data to suggest that the biological relationship between services per conception and the level of butterfat production is different from zero.

Artificial breeding associations would probably be more interested in total number of services as related to service interval rather than services per conception because, at the time of first service, no one really knows which cows will conceive and which will not. As shown in Equation (5), 0.56 less services can be expected for each 100 days of increase in the length of the service interval.

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DEVELOPMENT OF RUMEN FUNCTION IN THE DAIRY CALF. II. EFFECT OF DIET UPON CHARACTERISTICS OF THE RUMEN FLORA AND FAUNA OF YOUNG CALVES[1,2]

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SUMMARY

Twenty-four male calves were placed, at an average age of 3.5 days, on diets representing liberal and limited milk-feeding programs. The calves on limited milk were further divided into those receiving aureomycin in the concentrate mix and those not receiving it. In addition, a group of calves received only milk for 8 of the 12 wk. of the experiment. Rumen samples were taken weekly and analyzed for color, odor, consistency, total bacteria, aerobic bacteria, morphologically characteristic organisms, protozoa, the levels of niacin, biotin, and riboflavin, cellulose digestion, and the amounts of the lower fatty acids (C_2–C_4). For calves fed solid feeds, the general pattern of rumen function development seemed to be a sharp fall in the aerobic bacteria within the first 3 wk., an increase in the total numbers of bacteria, and the establishment of protozoa. The rumen acids were at adult levels by 6 wk. of age; whereas, the protozoal and bacterial numbers approached this point a little later. The establishment of adult-type organisms and cellulose digestion were slowest to develop and were hindered by aureomycin and liberal milk feeding. Biotin, niacin, and riboflavin levels increased up to 7 wk. of age, with an indication of synthesis early in life.

Much effort has been given to exploring the possibility of fostering an adult-like flora and fauna in the rumens of very young calves by the use of cud inoculations and/or high-roughage diets (4, 5, 8, 9, 10, 17, 18). To properly evaluate such programs, it seemed necessary to describe the establishment of the rumen organisms in calves raised under conditions of management and feeding on the farm.

As in the more recent publications on this subject (4, 5, 6, 8), it was felt that more criteria than the morphologically characteristic organisms would be needed. Therefore, it was decided to count the protozoa and bacteria and to assay for the ability to digest cellulose and the production of vitamins and fatty acids. In addition, the rumen characteristics of the adult animals in the herd were established (12), to serve as a base line.

METHODS

All samples were collected by stomach tube 6 hr. after feeding. After noting color, odor, and consistency, the rumen contents were strained through cheese cloth and the solid portion used for total bacterial counts, identification of

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characteristic organisms, aerobic plate counts, and the levels of niacin, biotin, and riboflavin. The liquid portion was used for protozoa counts, assay of fatty acids, and to test cellulose-digesting ability of the flora.

The fraction of the rumen contents, liquid or solid, used for a particular test, was chosen on the basis of where the numbers or concentrations could reasonably be expected to be greatest. Since the cellulolytic bacteria are intimately associated with the rumen solids, the solid portion was used for the identification and counting of bacteria. The bacteria and plant material are the sources of the vitamins; therefore, the solid portion was also used for the assay of the B-vitamins. The free swimming protozoa and the end-products of bacterial metabolism, the fatty acids, are found in the liquid portion, so this was used for their enumeration. In instances where no solids were obtained, the liquid was used instead.

Standard bacteriological techniques, described at length in a previous publication (12), were used in making slides for bacterial counts and for the plate counts of aerobic organisms. For identification of characteristic organisms, the system used by Rusoff *et al.* (19), to which was added a G^- spiral-like organism, 4 to 18 μ in length and 0.5 μ or less in width, was employed. The ability of the rumen organisms to produce gas from Solka-Floc was used as a criterion of cellulose digestion, as was a drop-weight technique developed by O'Neil *et al.* (16). Protozoa were counted by means of a Spencer Brightline hemocytometer (12). For the rumen acids, the method of Neish (15), as modified by Bauman (2), was employed. The method of Genghof *et al.* (7) was used to analyze for biotin, and the procedures of Bacharach and Cuthbertson (1) for riboflavin and niacin. The data were analyzed by means of the analysis of variance (20).

EXPERIMENTAL PROCEDURE

A total of 24 male calves—18 Holsteins, three Brown Swiss, two Guernseys, and one Ayrshire—were used for this study. The calves averaged 3.5 days of age and had not consumed solid foods at the start of the experiment. The experiment started in September of 1952 and lasted through May of 1953. Six animals comprised a group, though all were not on trial at the same time. Each animal was under observation for a period of 12 wk. As they were born in the herd, calves were assigned in rotation to the experimental groups.

The objective was to compare the development of the rumen flora and fauna when milk was the only nutrient, when calves received liberal amounts of milk along with hay and grain, and when animals were on a limited milk-feeding program, with and without antibiotics.

The group of calves designated as normal received milk for the entire experimental period at the rate of 10% of the body weight, with a maximum of 10 lb. per day. A 14% crude protein concentrate composed of 700 lb. each of corn and oats, 400 lb. of bran, 200 lb. of linseed oil meal, and 20 lb. each of bone meal and salt was fed free-choice, with a maximum offering of 3 lb. per day. Water and hay were supplied ad lib. In this and all other cases where hay was fed, a clover-timothy mixed hay of fair quality was used.

Two groups were placed on a limited milk program comparable to the system commonly used for raising calves for herd replacements. They received milk at the rate of 10% of the body weight during the first 3 wk. After this period, the milk was gradually withdrawn from the diet and no milk was offered after the end of the 7th wk.

Concentrate consisting of 480 lb. corn, 500 lb. linseed oil meal, 400 lb. corn gluten meal, 200 lb. each of soybean oil meal, oats, and wheat bran, and 20 lb. salt, approximately 25% crude protein, was fed free-choice with a limit of 3 lb. per day. One of these groups was designated as nonaureo and received the grain ration without additions. For the other group, called aureo, 40 g. of crystalline aureomycin-hydrochloride were mixed into each ton of concentrate. Water and hay were fed ad lib.

The fourth group of calves, the milk group, for the first 8 wk. received only milk, at the rate of 12% of the body weight per day. At the start of the 9th wk., the 14% crude protein concentrate and hay were offered. The milk was then rapidly reduced, so that no milk was offered by the end of the 11th wk.

All calves were kept in elevated mesh-floored tie stalls for the entire 12-wk. experimental period. They were housed in the same barn as adult cattle, but care was exercised to prevent contact or contamination. They were fed at 7 A.M. and 4 P.M. each day. Rumen samples were collected by stomach tube once a week at 1 P.M.

RESULTS

The rumen samples indicated that the calves which had the opportunity consumed appreciable amounts of solid feeds as early as 2 wk. of age. The rumen contents closely resembled those of adult cattle in color, odor, and consistency by 3 and 4 wk. of age, for calves on the limited and liberal milk feeding programs, respectively. The calves receiving only milk manifested a craving for dry feed at 2 wk. of age, and at 8 wk., when first offered hay and concentrate, avidly consumed from 3 to 5 lb. of hay per day.

With time, a definite increase in the number of varieties of microorganisms in the rumen was noted (Table 1). In the milk group, only a few types were present until the feeding of hay and grain began. When supplied with solid feeds it took but 3 wk. to produce a flora as diversified as that of the adult (12). Few differences were observed between normal and nonaureo groups, which represented liberal and limited milk-feeding programs, respectively. A flora as varied as that of the adult animals in this herd had developed in these calves by the 8th wk. The aureo group consistently showed a relative lack of organisms classified as Hay IIa and Spirals.

Counts of the rumen bacteria indicated that the number of organisms increased as the calves grew older (Figure 1). For calves receiving only milk, the bacterial count remained low until hay and concentrate were supplied. The numbers then increased markedly and at the 10th wk., 2 wk. after being offered solid foods, the bacterial count was equal to that of the other groups. For reference purposes, adult cattle of this herd had been observed (12) to have about 66 billion organisms per gram of rumen content.

TABLE 1

Influence of diet on the development of characteristic organisms in the rumen of the calf[a]

Week	Milk						Normal						Aureo						Nonaureo					
	Hay I	Hay II[a]	Grain I	Grain II	Misc. I	Spiral	Hay I	Hay II[a]	Grain I	Grain II	Misc. I	Spiral	Hay I	Hay II[a]	Grain I	Grain II	Misc. I	Spiral	Hay I	Hay II[a]	Grain I	Grain II	Misc. I	Spiral
0	0	1	0	6	2	0	0	0	0	6	4	0	0	0	0	5	4	2	0	0	2	5	4	0
1	0	0	1	6	1	1	0	0	0	6	3	5	1	1	0	6	4	2	1	0	2	6	2	3
2	0	0	0	6	0	0	0	0	0	6	5	4	2	2	2	6	6	1	3	1	2	**6**	**6**	**4**
3	0	0	0	6	3	1	1	1	3	6	6	5	3	3	3	6	6	0	2	0	3	5	6	4
4	0	0	1	6	2	0	2	1	2	6	6	**5**	4	3	2	6	5	2	2	0	3	6	6	6
5	0	0	2	6	4	0	3	1	5	6	6	5	5	4	0	6	5	1	3	2	4	6	6	4
6	0	0	1	6	4	0	4	1	4	6	6	5	6	3	3	6	5	2	5	2	3	5	6	4
7	0	0	1	6	4	0	4	2	3	6	6	6	4	1	1	6	6	0	5	2	3	6	6	4
8	0	0	1	6	6	0	5	3	2	5	6	5	5	0	3	6	6	0	5	4	3	6	6	6
9	3	1	2	6	6	4[b]	4	5	2	5	6	6	5	0	4	6	6	1	6	4	5	6	6	6
10	3	1	4	6	6	5	5	2	1	6	6	6	6	0	3	6	6	1	6	4	1	6	**6**	**6**
11	4	2	4	6	6	6	5	3	0	6	6	4	6	0	0	6	6	1	6	4	4	6	6	6
12	5	0	6	6	6	6	5	3	0	6	6	6	6	0	1	**6**	**6**	2	6	3	3	6	6	6

[a] A group consisted of six animals. Each figure in the table indicates the number of animals showing the organism.

[b] Hay and concentrate feeding initiated; milk reduced.

The numbers of aerobic bacteria showed marked changes during the course of the experiment (Figure 1). Most striking was the presence of approximately 240 million per gram of rumen contents at the first sampling. A sharp drop in numbers occurred during the 1st wk. of the experiment. In animals receiving solid foods, this new level tended to be maintained for another week and then decreased again to about three million bacteria per gram of rumen solids. This was lower than the ten million aerobes per gram of rumen solids noted for adult cattle. No differences were observed between normal and nonaureo groups; but after the 4th wk. the group fed aureomycin consistently showed about half the number of aerobic bacteria that the nonaureo group of calves showed.

Those animals maintained solely on milk showed very high levels of aerobic bacteria in the rumen contents. The lack of fibrous solid material, necessitating in some cases the use of liquid ingesta for bacterial counts, may have been a contributing factor to the differences in aerobe numbers. When supplied with solid foods, these animals followed the pattern of the other groups.

As seen in Figure 1, protozoa were established within the rumen during the 1st wk. on experiment. For calves receiving aureomycin, the numbers increased rapidly and equaled the adult level (477,000 per milliliter) at about 5 wk. of age. The nonaureo group exhibited a 2-wk. lag when compared to the aureo animals; however, both groups reached a similar peak at 11 wk. of age. The normal calves, receiving milk throughout the experiment, showed a slow development of the fauna and did not attain the numbers seen in adult cattle until 9 wk., and never approached the values for the nonaureo or aureo calves.

Most striking was the relative lack of a protozoan population in calves receiving only milk. These animals maintained a low count during the 9 wk. when only milk was fed, and for about 3 wk. after hay and grain feeding was begun. At 12 wk., the protozoa began to increase in number, a situation similar

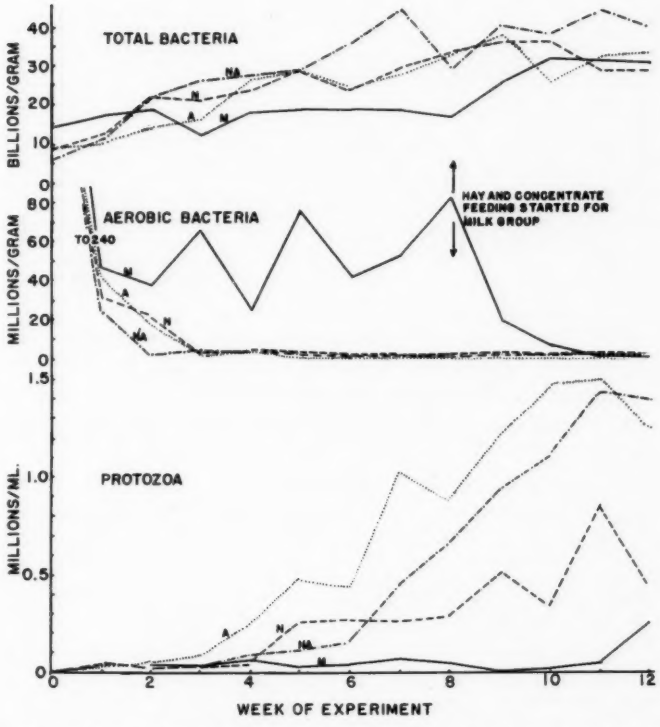

FIG. 1. The numbers of all bacteria, aerobic bacteria, and protozoa found in the rumen contents of calves raised under different regimes. For this figure, N indicates the normal group, M the milk, A the aureomycin, and NA the nonaureomycin group.

to that for 4-wk.-old calves fed hay and grain from the start. Thus, hay and concentrate seem to be necessary for the normal proliferation of the protozoan population.

Differences in the characteristics of the protozoan population were followed, as well. Only small ($< 5\,\mu$ in length) protozoans were present in the rumen of animals receiving only milk. When solid feeds were provided, the large ($> 5\,\mu$ in length) protozoa appeared. The aureomycin-fed calves exhibited the large protozoa in the rumen contents in all cases after 6 wk. on experiment; this was true for the nonaureo group at 11 wk. In the normal group, five of the six calves showed the presence of large protozoa at the 12th experimental week. In all calves, only a few heterotrichous types of protozoa (large) developed. Two calves receiving cud-inoculum developed a protozoan population which contained numerous varieties of both holotrichous and heterotrichous species.

Attempts were made to estimate the cellulolytic activity of the rumen flora. The criteria were the time to digest a cotton thread and the production of gas

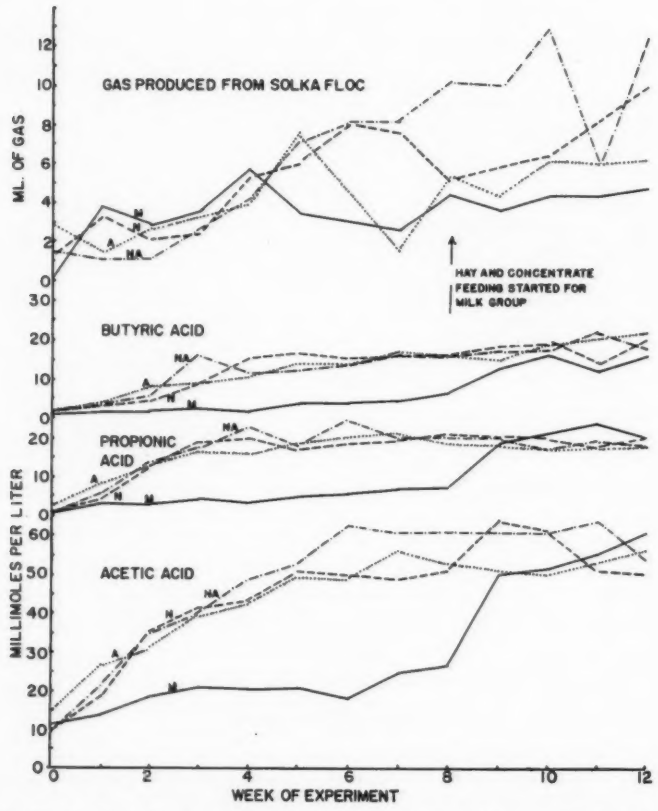

FIG. 2. The in vitro digestion of cellulose and the fatty acid concentration of the rumen contents of calves kept under different dietary conditions. For this figure, N indicates the normal group, M the milk, A the aureomycin, and NA the nonaureomycin group.

as a result of bacterial attack upon Solka-Floc. The data for the latter technique are presented in Figure 2.

In all groups, a gradual increase in cellulolytic activity was noted up to 4 wk. of age. At this time the animals fed only milk plateaued and never reached levels comparable to the other dietary groups. The aureo calves leveled off at 5 wk., apparently due to the inhibitory effect of aureomycin, at a value about one-half that for adult cattle (9.4 ml. of gas) and significantly lower ($P < 0.05$) than the nonaureo calves at Weeks 9, 10, and 12. The nonaureo and normal groups approached the adult level of activity at 6 wk. of age.

The test employing the breakage of a cotton thread indicated that the nonaureo calves were equivalent to adult cattle at 9 wk. of age. Aureomycin and milk feeding seemed to be inhibitory; however, it must be noted that even the rumen contents of the milk-fed group were capable of digesting cellulose.

Weekly analyses of the rumen levels of various acids were made throughout the experiment; the data are shown in Figure 2. Most striking are the low levels of butyric, propionic, and acetic acids found in the rumen contents of the milk-fed group during the period of milk feeding. This group was statistically different ($P < 0.05$) from all the other groups from Weeks 2 through 9. With the start of hay and concentrate feeding, the levels of the acids rose rapidly to become indistinguishable from those of the other groups. It should be noted that the rise at the 9th wk. was much sharper than the rises shown in the early weeks by those animals receiving hay and concentrate from the start. The difference possibly can be attributed to the comparatively large amounts of hay and concentrate consumed by the milk-fed calves when their diet was changed.

For the other dietary groups, few differences were noted. In general, these groups showed levels of butyric acid comparable to the adult (14.5 m*M*/liter) at 4 wk. For propionic acid, the adult value (18.1 m*M*/liter) was reached at the 3rd wk.; the acetic acid values took longer to plateau, but were at levels similar to those for adult cattle (63 m*M*/liter) at 6 or 7 wk. of age.

The formic acid content of the rumen material showed little variation, the values hovering around the levels established for adult animals (1.3 m*M*/liter). The milk-fed calves remained somewhat below this value ($\cong$ 0.8 m*M*/liter) and at the 2nd, 4th, and 6th wk. were found ($P < 0.05$) to be different from all other groups.

The succinic-lactic acid in the rumen appeared to vary widely around the level found for adult cattle (5.24 m*M*/liter) and few differences were observed. A tendency was seen for this group of acids to be high (10 to 30 m*M*/liter) during the early weeks and then to fall off. Only the nonaureo group showed that the values for Weeks 1 and 2 were significantly higher ($P < 0.05$) than for Weeks 3, 6, and thereafter.

Expressing the data of each acid as per cent of the total of the rumen acids titrated, it was noted that for the milk-fed animals the per cent of acid was significantly lower ($P < 0.05$) at Weeks 7 and 8 for butyric and at Weeks 3 through 7 for propionic, than for calves of the other groups. Conversely, the proportions were greater ($P < 0.05$) for acetic at Weeks 2, 3, 4, and 6 and for formic at Weeks 5, 6, and 7. For the succinic-lactic acids, no real differences were seen between groups; however, this fraction formed a large portion of the rumen acids during the first sampling period and was lower ($P < 0.05$) after 3 wk. In the first sampling period, the succinic-lactic fraction averaged 41% of all the acids titrated; whereas, at 12 wk. it represented but 5.3%.

The levels of some of the B-vitamins in the rumen solids were determined and are shown in Figure 3. For biotin, no significant differences could be detected, though the aureomycin-fed calves consistently averaged higher than other groups. The aureo group showed a trend for the levels of biotin in the rumen to increase during the experimental period. Statistically, the levels at Weeks 1 and 2 were lower ($P < 0.05$) than those for Weeks 7 and thereafter.

The data for niacin (Figure 3) showed no differences between groups, though the aureomycin-fed calves, during Weeks 5 through 11, consistently averaged

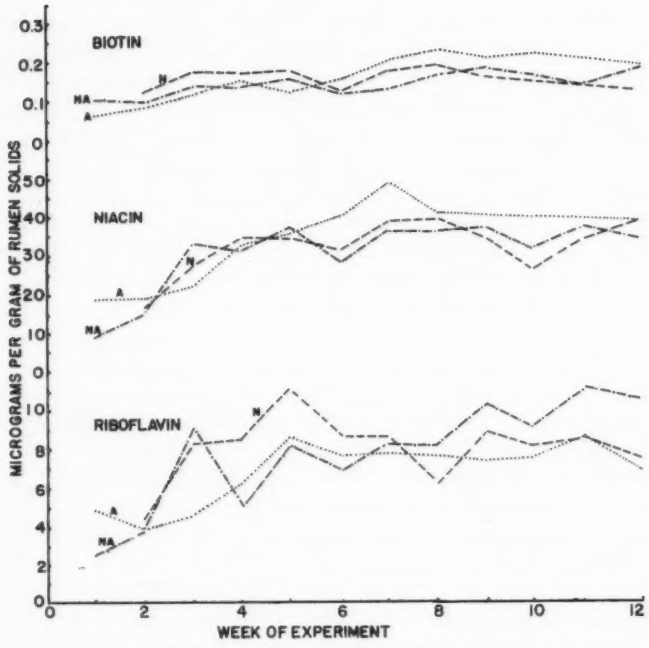

FIG. 3. The concentration of some B-vitamins in the rumen fluid of calves raised under various feeding systems. For this figure, N indicates the normal group, A the aureomycin, and NA the nonaureomycin group.

higher. Only in this group could an indication of an increase with age be shown. The value for Week 1 was lower ($P < 0.05$) than the values for weeks thereafter; Week 2 differed from Weeks 5 and thereafter; and Week 3 differed from weeks thereafter.

For riboflavin (Figure 3), no differences existed between groups. Some suggestion of an increase in the levels of riboflavin in the contents could be seen; however, this could be shown statistically for the nonaureo group only. For these animals, Week 1 differed ($P < 0.05$) from Weeks 9 and thereafter; Week 2 differed from Weeks 9, 11, and 12.

For comparative purposes, the concentrates fed were found to contain 0, 4.5, and 0.013 γ/g of dry matter of riboflavin, niacin, and biotin, respectively. The hay contained 4.8, 12.2, and 0.010 γ/g of dry matter of riboflavin, niacin, and biotin, respectively. Milk contained 0.96, 2.89, and 0.025 γ/g of riboflavin, niacin, and biotin, respectively. The solids obtained from the rumen contained ten times as much biotin at the 2nd wk. of age as was present in the concentrate or hay. The niacin levels were two to three times those of the roughage, and for riboflavin were about one and one-half times richer. Since the rumen liquid was also certain to contain some of these vitamins, this is evidence of very early synthesis of these vitamins in the rumen of the calf.

DISCUSSION

These experiments indicate that functional development of the rumen, similar to that seen in mature animals, can be attained at very young ages in dairy calves by using standard feeding practices. Few differences attributable to alterations in the feeding program could be seen among calves receiving hay and concentrate.

Using the nonaureo group as being indicative of the rumen function development of calves raised on a limited milk-feeding program without antibiotics, it was seen that the rumen acids had reached adult levels, the vitamin content of the rumen solids had plateaued, and cellulose digestion (gas-production method) could not be distinguished from the adult by the 6th wk. of the experiment. The numbers and types of bacteria and protozoa took longer to develop, but resembled the adult at the 8th wk.

This group of calves thus closely resembled the cud-inoculated calves of Bryant and Small (4) and calves fed high-roughage pellets by Conrad *et al.* (6). The most striking difference was the development of a protozoan population [the uninoculated calves of Conrad *et al.* (6) failed to have this development]. The population was not as diverse as that of two cud-inoculated calves or the calves of Bryant and Small (4). It is evident that some contamination from the adult animals did occur, even though there was no direct contact or use of common utensils. This indicates that when calves are kept in the proximity of mature animals, special measures to provide them with cud-inoculum need not be taken. This is supported by the finding of no significant differences in the performance of uninoculated calves on high-roughage diets, when compared to those receiving rumen inoculum (8).

A liberal milk-feeding program, as represented by the normal group, seemed to delay the acquisition of some adult characteristics. In most cases, adult values were reached from 1 to 2 wk. later than in the nonaureo calves. By 9 wk., the rumen function was equal to that of adult cattle in all respects except the digestion of cellulose. By the 12th wk., even this goal was attained. It is to be noted that the nonaureo and normal groups acted alike up to 6 wk. of age, then differed. The rapid withdrawal of milk from the diet of the nonaureo group seemed to accelerate the maturation of the rumen function. Milk has been noted to have a depressing effect upon the size of the rumen protozoal population (13) and may explain the marked increase in the numbers noted in the nonaureo group when milk was withdrawn; whereas, a relatively slow increase took place in the normal calves. Somewhat the same picture was presented for the cellulose-digesting ability of the rumen liquor.

Aureomycin had an enhancing effect, in that the protozoal population seemed to be fostered, which agrees with the data of Mann *et al.* (14), and the biotin and niacin levels of the rumen contents were higher than observed for the other groups. However, the aureomycin did have an inhibitory effect on the microorganisms of the rumen. Of the indicator organisms, the Hay IIa, confirming Hibbs and Conrad (9), and the Spiral, were lacking. Also, it was noted that the rumen contained fewer aerobic bacteria than did the other groups. From this, it

can then be surmised that other, less distinctive types of organisms were affected, as well. This probably contributed to the lessened ability to digest cellulose, an observation also noted by some others (3, 6, 11).

The calves fed only milk showed a rumen function that was markedly immature when compared to the other animals; however, some cellulolytic activity was present (5). The supplying of hay and grain brought about the immediate appearance of new organisms which were more characteristic of adult rumen contents; the amounts and percentages of the rumen acids also came to resemble those of the other groups more closely. The results obtained with this group, and the less dramatic differences seen between the other groups, lead to the conclusion that the major factor affecting rumen function development was the diet. All animals on a diet containing hay and concentrate rapidly developed the same characteristics of rumen population as seen in the adult animals of this herd. Thus, it is evident that inoculation occurs as a natural event and that the fostering of such a population is mostly a problem of management of the substrate presented to the organisms, and that feeding practices now in use are successful in promoting early functional development.

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EFFECT OF ETHANOL ON RUMEN FERMENTATION[1]

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SUMMARY

Ethanol (800 ml., 47.5%) was administered via rumen fistula during the once-daily feeding of: (1) 14 lb. timothy hay plus 4 lb. of equal parts of ground corn and ground corncobs or (2) Ration 1 with 3% urea replacing corn or (3) 20 lb. alfalfa hay, to twin 1,100-lb. cows. Each ration was fed with and without ethanol to each cow for 14-day periods. Four per cent of ground ration was added to cheese cloth–strained rumen fluid and incubated for 3 hr. in vitro. Ethanol was not fermented in vitro but disappeared from the rumen with a half-time of 2 hr. Blood contained 75 mg.% ethanol 2 to 4 hr. after administration. Ethanol depressed rumen fill, gas production in vitro, and the utilization of radioactive sulfate for synthesis of amino acids and glutathione, but had little effect on the volatile fatty acid concentration in the rumen. Digestibility of crude protein and organic matter was slightly reduced by feeding ethanol with grain rations (1) and (2) to four dry nonfistulated cows.

Recent reports on the value of adding ethanol to rations for growing beef heifers (8), fattening cattle (9), growing dairy heifers (2), and lactating cows (1) have been inconclusive. Tsuda (11) has reported that ethanol is rapidly absorbed from the rumen. The present study was initiated to determine the effect of ethanol on rumen fermentation and to investigate its absorption from the rumen.

EXPERIMENTAL PROCEDURE

Twin 1,100-lb., dry Holstein cows, equipped with rumen fistulas, were fed the following ration once daily: 14 lb. of timothy hay plus 4 lb. of a grain mixture, consisting of equal parts of ground corn and ground corncobs plus salt and minerals (Ration 1). When 3% urea was added to this ration, the proportion of corn and corncobs was altered to maintain an equal TDN (Ration 2). Ration 3 consisted of 20 lb. of alfalfa hay. One twin received Ration 1 followed at 14-day intervals by Ration 1 plus ethanol, Ration 2 plus ethanol, and then Ration 2. Six months later, this same cow was fed Ration 3 plus ethanol for 21 days, followed by Ration 3 for 21 days. During these same 14- and 21-day periods, the other twin was fed successively Ration 1 plus ethanol, Ration 1, Ration 2, Ration 2 plus ethanol, Ration 3, and Ration 3 plus ethanol. Blood and rumen samples were taken 0, 2, and 4 hr. after feeding at the end of each period.

When Rations 1 and 2 were fed, the rumens were emptied of material on the last day of each period, 4 hr. after feeding. When ethanol was administered, 800 ml. of 47.5% alcohol was poured into the rumen fistula at the time of feeding and mixed with the contents. For the in vitro fermentations, a 3.5-hr. incubation period was used for the determination of gas production. Inoculum consisted of cheese cloth–strained rumen fluid. A 25-ml. portion was used for the ammonia

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and sulfate incorporation trials and a 20-ml. portion was used for the determination of gas production. The substrate level was 2 to 4 g. per 100 g. of inocula and consisted either of ground corn, of ground hay, or of combinations of the two. Where ammonium acetate was added, the level was 0.1 g. per 100-ml. inoculum. These fermentations were carried out in stoppered Erlenmeyer flasks in a 39° C. water bath.

Ammonia formation was determined in 36 flasks, of which 18 were controls and 18 contained 500 mg. % of ethanol. Three incubation times of 0, 1.5, and 3 hr. were used with each of three substrates: corn, corn plus ammonium acetate, and hay plus ammonium acetate. Two of the four flasks in each substrate-time subgroup contained inoculum from one twin; the two other flasks contained inoculum from the other twin. One of the two flasks in each set contained ethanol. Incorporation of inorganic sulfate was determined in 16 flasks. Two control flasks and two flasks with 500 mg. % ethanol were used for each of the twin cows when they received the hay ration. The entire trial was repeated when the hay ration plus ethanol was fed. Each flask contained 25 ml. inoculum, 0.25 g. corn, 0.25 g. hay, and 250 μc. of sulfur^{35}. Incubation time was 3 hr. Statistical significance was determined by analysis of variance.

The Keeney column (6) was used for the determination of volatile fatty acids, aeration for the determination of ammonia, aeration plus urease for the determination of urea, and a micro-diffusion method (7) for the determination of blood and rumen alcohol. The microbial amino acids and free glutathione were separated, identified, and assayed as described by Emery *et al.* (4).

Digestion and nitrogen balance trials were conducted with four successive ten-day preliminary and seven-day collection periods. In each period, four rations consisting of 12 lb. timothy hay plus 6 lb. of the corncob-corn mixture were fed alone, or with urea, or ethanol, or urea plus ethanol. The same four nonfistulated dry cows were used in all periods and were assigned to the rations at random. These rations were fed in equal portions, morning and evening. The ethanol also was fed twice daily by pouring it on the grain at feeding time.

RESULTS AND DISCUSSION

The mean concentration of blood alcohol 2 to 4 hr. after administration via the rumen fistula was 75 mg. %. The rumen concentrations 1, 2, and 4 hr. after administration of the ethanol were: 369 mg. %, 265 mg. %, and 145 mg. %, respectively. If these values are plotted on semilog paper and extrapolated back to the time of administration, and the average rumen volume as measured by emptying the rumen is taken into account, one finds that the maximum theoretical concentration of rumen ethanol was 500 mg. %. This alcohol concentration (500 mg. %) was then used for the in vitro fermentations when measuring ammonia metabolism and sulfate incorporation into amino acids. When gas production was determined, the ethanol naturally present in the inoculum as taken from the animal at about 2 hr. after feeding was sometimes used without further addition of alcohol. The concentration of rumen ammonia did not change with the administration of ethanol, although it was significantly affected by ration

TABLE 1
Ammonia nitrogen and fatty acids in rumen contents

	Rations					
	Corn-Corncobs-Timothy		Corn-Corncobs Timothy-Urea		Alfalfa hay	
	Control[a]	Ethanol[a]	Control[a]	Ethanol[a]	Control[a]	Ethanol[a]
Acetic Acid *(meq/l)*	56.8	63.8	67.5	66.3	99.9	98.4
Propionic Acid *(meq/l)*	14.3	16.6	18.6	16.3	28.0	28.7
Butyric Acid *(meq/l)*	8.2	11.2	9.7	9.2	15.6	12.7
Valeric Acid[b] *(meq/l)*	1.2	1.5	1.1	1.8	3.0	3.6
"Higher" acids *(meq/l)*	0.4	0.4	0.6	0.6	0.6	0.9
Total acids *(meq/l)*	80.9	93.5	97.5	94.2	147.1	144.3
Ammonium-N[c] *(mg. %)*	5.7	5.4	10.6	13.3	26.6	26.7

[a] Two samples per subgroup for acids, six for ammonia-N.
[b] P = about 0.3 for ethanol vs. control, combined rations.
[c] Mean for samples taken 0, 2, and 4 hr. after feeding.

(Table 1) and time after feeding. Ethanol tended to depress the concentration of ammonia nitrogen in the flasks fermented in vitro, as shown in Table 2. Ammonia nitrogen increased with fermentation time in these flasks. Therefore, there was little or no net synthesis of protein, and ethanol apparently depressed ammonia formation. When the incorporation of sulfate sulfur^{35} was utilized as a measure of protein synthesis (Table 3), it was found that methionine,

TABLE 2
Ammonia formation during in vitro fermentation

Substrates	Control	Ethanol
	(mg. % NH_3-N in media)	
Corn	10.4	10.2
Corn + ammonium acetate	22.7	22.2
Hay + ammonium acetate	52.1	51.9
Mean of all substrates	28.4	28.1[a]

[a] P less than 10%.

cystine, and/or cysteine and glutathione synthesis were all slightly depressed by the presence of ethanol. In cystine formation, this was significant at the 5% level.

Ethanol depressed blood urea nitrogen in the trials with the fistulated cows from a mean of 32.9 mg. % for the control to 30.6 mg. %. These cows were emaciated throughout the experiment. A mean blood urea-N content of 8.4 mg. % was observed with the ethanol rations in the balance trial versus 6.8 mg. % with the control rations. Both differences approached significance at the 5% con-

TABLE 3
Microcuries inorganic S^{35}–sulfate incorporated into amino acids

	Methionine[a]	Cysteine[a]	Glutathione[a]
Ethanol	2.4	11.0[b]	6.4
Control	3.4	14.9	7.6

[a] Compounds plus their decomposition products.
[b] Significantly lower than control at 5% level.

fidence level. Bates *et al.* (2) found that 6% ethanol in molasses augmented blood urea when fed ad libitum. The total ethanol ingestion under the conditions of Bates *et al.* (2) was about one-half that in the trials reported here. The inconsistency in blood urea might be due either to the method of administration of the ethanol or to the emaciation noted in the fistulated cows.

The total gas production in vitro was depressed by ethanol in each of the five trials (Table 4). Gas was collected for the 1st hr., then was collected separately for the last 2 hr. of this fermentation. The CO_2 to methane ratio was always much higher for the 1st hr. than for the last part of the fermentation. Ethanol tended to depress methane slightly more than CO_2 production. This tendency occurred only during the last part of the fermentation and was not significant.

Although ethanol disappears from the rumen with a half-time of about 2 hr., when the ethanol concentration was determined during in vitro fermentations before and after incubation, there were no indications of a disappearance of ethanol. Therefore, ethanol was not fermented by the rumen bacteria. This is surprising, since it is known that bacterial species present in the rumen do utilize ethanol for growth (3) and energy (10) under certain circumstances. It would appear that the ethanol did affect the metabolism of the rumen bacteria, particularly when it was in concentrations of 500 mg. %. However, at the level fed, this concentration was present in the rumen only a very short time, if at all.

TABLE 4

Ratio of CO_2 to CH_4 in gas produced during 3.5 hr. fermentation of rumen material in vitro[a]

	Control	Ethanol	Probability[b]
Total gas *(ml.)*	272	225	15%
Ratio CO_2/CH_4	4.6	4.8	>50%

[a] 250 mg. % to 500 mg. % ethanol in media.
[b] Five trials, probability of a larger F to the nearest 5%.

Where effects on metabolism were noted, particularly in the gas production, the effect of the ethanol was to decrease fermentation rates. Bates (2) observed a slight depression of the rumen volatile fatty acids with the exception of valeric acid by ethanol feeding; Head (5) observed an augmentation of the rumen acetic acid concentration with ethanol feeding. The results of this experiment (Table 1) confirm those of Bates (2). Ethanol also tended to depress the amount of digesta in the rumen. The mean amounts of wet digesta expressed as percentage of body weight were 16.0 for the control and 14.6 for the ethanol-treated cows. On the dry basis, these values were reduced to 1.9% for the control and 1.8% for the ethanol-treated cows.

Ethanol tended to depress digestibility and nitrogen retention (Table 5). These effects were not significant, although the depression of crude protein digestibility approached significance at the 5% confidence level. These results are in agreement with those of Balch *et al.* (1).

TABLE 5

Results of digestibility and nitrogen balance trials

	Control		Ethanol	
	Low protein	Urea	Low protein	Urea
Digestibility *(%)*				
Organic matter	64.0	67.2	62.2	65.5
Crude protein	51.2	65.9	45.6	64.5
Nitrogen balance				
Daily intake *(g.)*	87.8	124.1	87.8	124.1
Daily retention *(g.)*	25.6	48.6	19.2	50.6

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EXTENT OF DIGESTION IN THE RUMEN-RETICULUM-OMASUM OF GOATS

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SUMMARY

The extent of digestion in the rumen-reticulum-omasum was studied, using goats whose forestomaehs were separated from the abomasum, in order to determine quantitatively the amount of omasal ingesta. Eleven digestion trials were conducted using three such forestomaeh-separated goats giving various kinds of feeds, e.g. roughage alone, hay with various concentrates, and a nonprotein diet.

The absorption of digestible organic matter from the forestomaeh ranged from 61.7 to 85.4%. The apparent digestibility coefficients of crude protein in the forestomaeh varied widely and were positively related to dietary protein levels. The absorption of digestible protein in the forestomaeh ranged from −20.0 to 52.1%. In animals fed roughage alone, 61.9 to 73.2% of digestible ether-extract disappeared from the forestomaeh but, in animals fed concentrates and hay, smaller amounts (−2.9 to 28.8%) were absorbed from the forestomaeh. Almost all of the digestible crude fiber disappeared from the forestomaeh and 65.7–96.9% of the digestible NFE was absorbed from the forestomaeh. Except in one trial, crude ash showed negative values, due to the large amount of saliva secreted into the rumen.

The true digestibility of protein in the forestomaeh, and the amount of saliva secreted, also were studied.

The importance of the digestion processes taking place in the rumen has long been recognized but, as yet, there are few quantitative data available. In previous work, samples of rumen contents for subsequent analysis have been obtained by complete removal of the rumen contents from fistulated animals at various time intervals (7, 8), from samples of the reticulo-rumen digesta taken intermittently near the omasal orifice of fistulated animals (1), or by slaughter of the animal at a predetermined time after feeding and subsequent sampling of the gut contents (5). The above procedures included the use either of external or of internal digestibility indicators, chromic oxide or lignin. Because of the varying rates of passage of these indicators through the alimentary tract (10, 11, 12), the data are subject to uncertain errors. Therefore, it would seem more desirable, in estimating the extent of digestion in the rumen, to quantitatively collect alimentary tract contents upon entry into the omasum or abomasum, by establishment of an appropriate fistula. Such a fistula appeared feasible in light of the recent report by Conver *et al.* (2), but their duodenal fistula would not be appropriate for studies dealing with rumen digestibility.

The present work was concerned with quantitative estimation of rumen digestibility in goats by collecting all alimentary tract contents upon entry into the abomsum via a fistula establishment between the abomasum and omasum.

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EXPERIMENTAL PROCEDURE

Animal. Three female goats of a native Japanese breed were used. This is a light-weight, early-maturing breed and the animals are easy to manage after an operation. Their ages were 3 yr. (Goat A) and 18 mo. (Goats B and C) and their body weights were 30, 22, and 21 kg., respectively, at the start of this experiment. Female goats were used, since they were suitable for the separate collection of feces, urine, and the omasal ingesta, as the new outlet of the omasum was located on the ventral surface of the body.

Surgery. After two days of fasting, a goat was anesthetized by intravenous injection of 10 ml. of 0.4% of Rabonal.[1] An incision about 15 to 20 cm. was made along the mid-line, and the juncture of the omasum and abomasum was withdrawn from the body cavity. The omasum was cut from the abomasum, avoiding the main blood vessels and nerves. A new outlet for the omasum about 1 cm. in diameter was located and sutured along the mid-line. After completely suturing the open end of the abomasum, it was located and sutured to the right abdominal wall about 15 cm. from the mid-line. A second-stage operation was performed two days later, to make a new inlet for the abomasum. A polyvinyl chloride plastic pipe, 1 cm. in diameter, was inserted into the abomasum through which omasal ingesta and other nutrients were to be introduced (Figure 1). The length of the pipe to stay inside daily varied from 4 to 8 cm., to avoid the continuous stimulation on the same spot of the abomasum wall. After opening the

[1] Rabonal contains 0.5 g. of thiopental sodium per gram.

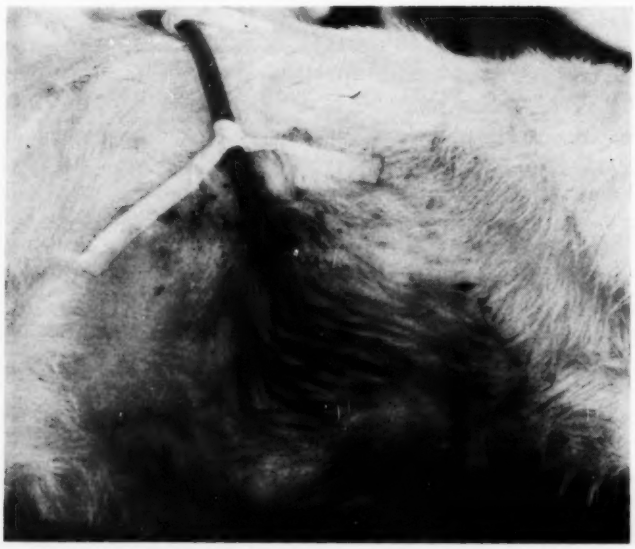

FIG. 1. Omasal outlet and abomasal inlet with vinyl pipe.

new inlet, the feed and water were given gradually. In this report, the goats on which this operation was performed were called the forestomach-separated goats. Further details of the operation will be reported in a separate paper.

Management of the forestomach-separated goats. The goats recovered health and appetite after about seven days, when they were equipped with the apparatus for collecting the omasal ingesta (Figure 2) and placed in metabolism cages. To aid in the total collection of omasal ingesta, the movement of the goats was restricted to a minimum. It was observed that the omasal content did not flow out from the omasal outlet but was pushed out by the action of the omasum.

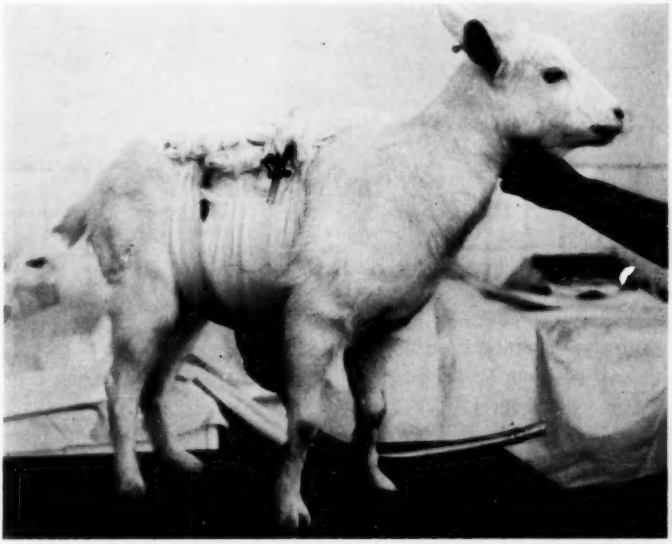

FIG. 2. The forestomach-separated goat with apparatus for collection of the omasal ingesta.

So the words omasal ingesta in this report do not mean the omasal content but the material which has completely gone through the digestive process in the forestomach. Omasal ingesta was poured into the beaker placed under the metabolism cage. The amount was weighed hourly, warmed at 40° C., and poured into the abomasum through the vinyl pipe. Intervals longer than an hour caused the feces to become soft and a tendency for diarrhea to develop. The apparatus for collecting omasal ingesta was renewed once a day.

Digestion trials. Digestion trials were conducted as in the conventional method, except in the treatment of the omasal ingesta. The management of the forestomach-separated goats was so tedious that the digestion trials were conducted continuously, in the order shown in Table 1. The periods of prefeeding and feces collection were shortened to seven days, except in Trial 11, when the prefeeding period was six days and feces collection period was four days. During

TABLE 1

Design and duration of experiment, weights of feed, and omasal ingesta and feces produced during the collection period

Trial No.	Goat	Pre-feeding	Collection	Feed consumed[a]		Omasal ingesta Fresh	Omasal ingesta Dry[a]	Feces[a, b]
		(days)	*(days)*		*(g.)*			
1	A	7	7	Conc.	1,538	33,684	1,908	1,345
				Hay	1,834			
2	A	7	7	Rye (green fodder)	4,002	41,713	1,939	1,135
3	B	7	7	Conc.	1,425	26,244	2,203	1,332
				Hay	1,757			
4	C	7	7	Conc.	1,741	28,678	2,204	1,353
				Hay	1,757			
5	B	7	7	Hay	2,542	26,491	1,580	1,273
6	C	7	7	Hay	2,839	30,514	1,878	1,422
7	B	7	6	Conc.	639	18,204	1,182	693
				Hay	1,294			
8	C	7	6	Conc.	2,234	26,575	1,710	940
				Hay	965			
9	B	7	7	Conc.	960	27,420	1,951	1,340
				Hay	1,974			
10	C	7	7	Conc.	1,089	30,092	2,061	1,553
				Hay	2,238			
11	B	6	4	Conc.	761	9,136	358	138

[a] Dry matter.
[b] These values were corrected with calculation.

the digestion trials, the sampling was performed hourly 24 times a day; the omasal ingesta was collected in the beaker, which was mixed thoroughly every time before one-tenth of it was removed. These samples were dried immediately in a circulating air dryer at 55° C., then weighed for chemical analysis. It was the aim of the authors that these samples would be the same as those obtained by collecting the entire omasal ingesta throughout the test period, homogenizing, and taking one-tenth of it for a sample. The amounts of feces shown in Table 1 were adjusted by a factor of ten-ninths, so as to equal the amounts of feces obtained when the omasal ingesta was not removed.

In Trials 2, 5, and 6, the goats were fed on roughage only, and in Trial 11 they were fed a semisynthetic nonprotein ration. In all other trials, a low-quality hay of predominantly orchard grass and red clover was fed with various concentrate mixtures. The composition of the concentrate mixtures is given in Table 2. They were altered to obtain various fiber-protein-urea-carbohydrate ratios.

As the goats in Trials 5, 6, and 11 were given relatively little or no protein, 2 g. of casein was administered into the abomasum with the remainder of omasal ingesta hourly, i.e., 48 g. per day. In these trials, the digestibility coefficients of protein were calculated on the assumption that this casein was absorbed completely from the gut. Water was given ad libitum.

The entire feces was collected before the morning feeding, and the residues of feed and water were weighed and measured. A ration containing no nitrogen was given to a goat in Trial 11, which was performed to determine the true digestibility of the protein in the forestomach. The sources of endogenous nitro-

TABLE 2

Composition of concentrate mixtures fed during each specified trial

Ingredient	Trial No.					
	1	3 and 4	7 and 8	9	10	11
	(%)					
Wheat bran	35	35	—	47	47	—
Rice bran	35	30	—	—	47	—
Ground corn	17	10	—	47	—	—
Soybean meal[a]	10	10	—	—	—	—
$CaCO_3$	2	2	—	2.5	2.5	—
NaCl	1	1	—	1	1	—
Dried skimmilk	—	12	—	—	—	—
Casein	—	—	23	—	—	—
Sweet potato powder	—	—	30	—	—	—
Corn starch	—	—	30	—	—	40
Sucrose	—	—	7	—	—	28
Soybean oil	—	—	5	—	—	6
Mineral mixture[b]	—	—	5	—	—	5.9
Vitamin mixture[c]	—	—	—	—	—	0.1
Urea	—	—	—	2.5	2.5	—
Filter paper	—	—	—	—	—	20

[a] Solvent extracted.

[b] Mineral mixture $CaHPO_4 \cdot 2H_2O$ 11.0%; $CaCO_3$ 16.0%; K_2HPO_4 27.0%; $MgSO_4 \cdot 7H_2O$ 15.0%; Na_2SO_4 22.6%; $FeC_6H_5O_7$ 2.5%; $MnSO_4 \cdot 4H_2O$ 0.7%; KI 0.08%; $ZnCl_2$ 0.02%; $CuSO_4 \cdot 5H_2O$ 0.03%; $COSO_4 \cdot 7H_2O$ 0.07%.

[c] Each 1 g. contains vitamin A, 2,500 I.U., vitamin D_2, 200 I.U., vitamin E, 1.0 mg., vitamin B_{12}, 1 γ, and so on.

gen may be saliva, along with rumen-wall cellular material, realimentation of liquids, etc. The authors intended to attempt the estimation of saliva, definitely the major source of endogenous nitrogen, not counting the probable water secretion and absorption taking place there. The method of estimation was as follows: (fresh weight of ingesta passing through the omasum) − (weight of water taken plus dry weight of omasal ingesta).

Chemical analysis. Moisture, crude protein, ether-extract, crude fiber, NFE, and crude ash were determined by the A.O.A.C. methods.

RESULTS

The total amount of feed consumed, omasal ingesta and feces produced during the feces collection periods are shown in Table 1. The amount of feed consumed by each goat was variable between trials, due to the palatability of feed and the condition of the goats, though intake during each trial was nearly constant.

The results of chemical analyses of each feed, omasal ingesta, and feces are shown in Table 3. The contents of protein, ether-extract, and crude ash in omasal ingesta were always higher than the corresponding values of both feed and feces. The opposite tendency was shown for the content of organic matter, crude fiber, and NFE. These tendencies seem to mean that the former are chiefly absorbed from the hind gut, and the latter from the forestomach.

In these observations, the apparent digestibility coefficients of crude protein in the forestomachs varied widely from −8.2 to 38.5%, as shown in Table 4. The digestibility coefficients of ether-extract in the forestomachs were low or negative,

TABLE 3

Chemical compositions of feed, omasal ingesta, and feces in each digestion trial

Trial No.		Organic matter	Crude protein	Ether-extract	Crude fiber	NFE	Crude ash
		(%)					
	Feed	86.61	14.51	2.30	18.58	51.22	13.39
1	Omasal ingesta	74.85	24.16	4.01	12.90	33.78	25.15
	Feces	78.38	18.23	2.71	17.52	39.92	21.62
	Feed	90.14	24.78	5.09	21.81	38.46	9.86
2	Omasal ingesta	74.40	31.47	5.44	9.93	27.56	25.60
	Feces	84.77	22.80	6.14	15.58	40.25	15.23
	Feed	87.34	16.94	2.51	17.30	50.59	12.66
3	Omasal ingesta	78.29	22.10	3.70	12.31	40.18	21.71
	Feces	79.88	18.72	2.56	20.72	37.88	20.12
	Feed	87.71	17.64	2.53	16.30	51.24	12.29
4	Omasal ingesta	75.12	25.02	3.99	13.03	33.08	24.88
	Feces	79.30	20.01	2.42	20.64	36.24	20.70
	Feed	85.81	11.94	2.30	25.33	46.24	14.19
5	Omasal ingesta	76.96	19.26	2.57	18.12	37.01	23.04
	Feces	82.52	13.43	2.31	22.32	44.46	17.48
	Feed	85.81	11.94	2.30	25.33	46.24	14.19
6	Omasal ingesta	79.30	19.53	2.42	18.38	38.97	20.70
	Feces	82.71	14.09	2.51	22.29	43.82	17.29
	Feed	87.79	16.12	3.81	17.43	50.43	12.21
7	Omasal ingesta	77.10	23.17	5.72	12.24	35.97	22.90
	Feces	80.22	19.81	2.45	16.49	41.47	19.78
	Feed	89.98	20.71	5.46	8.74	55.07	10.02
8	Omasal ingesta	77.78	29.26	7.81	5.75	34.96	22.22
	Feces	81.70	26.87	3.20	10.90	40.73	18.30
	Feed	87.94	14.90	2.87	19.59	50.58	12.06
9	Omasal ingesta	77.23	22.61	3.71	14.52	36.39	22.77
	Feces	81.49	17.55	2.89	20.31	40.74	18.51
	Feed	85.92	16.31	2.55	20.89	46.17	14.08
10	Omasal ingesta	74.06	20.38	3.71	15.37	34.60	25.94
	Feces	79.80	15.66	2.57	21.47	40.10	20.20
	Feed	95.16	1.56	6.26	20.18	67.16	4.84
11	Omasal ingesta	76.09	15.67	7.56	20.69	32.17	23.91
	Feces	90.78	17.24	2.50	40.03	31.01	9.22

except when roughage was fed alone. For crude fiber, almost the same values were obtained in the forestomachs as in the entire gut. NFE was digested to a markedly greater degree in the forestomach than in the hind gut. Except in Trial 6, the digestibility coefficients of crude ash in the forestomachs were always negative, due to the large amount of salivary ash secreted into the rumen.

To understand clearly the extent of digestion in the forestomachs, the ratio of the digestibility coefficient of each component in the forestomach to that in the entire digestive tract is shown in Table 5 (61.7 to 85.4% of digestible organic matter disappeared in the forestomachs). The disappearance of digestible protein in the forestomachs ranged from −20.0 to 52.1%, except in the nonprotein feeding trial. When goats were fed either on roughage alone or on a nonprotein diet, 46.5 to 73.2% of digestible ether-extract disappeared from the forestomachs. In contrast, when they were fed on hay with various amounts of concentrate, lesser amounts were absorbed from the forestomachs, ranging from −2.9 to 28.8%. The disappearance of fiber in the forestomachs ranged from 93.0 to 104.6%.

To estimate the true digestibility of protein in the forestomach, the amount of saliva secreted was determined, and the results are shown in Table 6. The

TABLE 4

Digestibility coefficients of feed, in the forestomach and in the entire gut

Trial No.		Organic matter	Crude protein	Ether-extract	Crude fiber	NFE	Crude ash
		(%)					
1	Forestomach	51.1	5.8	1.4	60.7	62.7	−6.3
	Entire gut	63.9	49.9	53.0	62.4	77.2	35.6
2	Forestomach	60.0	38.5	48.2	78.0	65.3	−25.9
	Entire gut	73.4	73.9	65.8	79.8	70.3	56.2
3	Forestomach	38.1	10.0	−1.6	50.9	45.2	−18.2
	Entire gut	61.9	53.9	57.5	50.1	68.7	33.8
4	Forestomach	46.1	10.7	1.5	49.6	59.2	−27.6
	Entire gut	65.1	56.1	62.9	51.0	72.8	35.9
5	Forestomach	44.3	−0.2	30.8	55.5	50.2	−0.8
	Entire gut	51.9	43.7	49.8	55.9	51.9	38.4
6	Forestomach	38.9	−8.2	30.5	52.0	44.3	3.5
	Entire gut	51.7	40.9	45.9	55.9	52.5	26.4
7	Forestomach	43.6	7.7	3.6	54.9	54.3	−20.4
	Entire gut	66.0	59.0	79.8	54.8	70.3	33.5
8	Forestomach	53.8	24.7	23.9	64.5	66.1	−18.7
	Entire gut	73.3	62.0	82.9	63.1	78.3	46.3
9	Forestomach	41.6	−1.0	13.8	50.7	52.2	−25.6
	Entire gut	57.7	46.2	53.9	52.6	63.2	29.9
10	Forestomach	46.6	22.6	9.9	54.4	53.6	−14.1
	Entire gut	56.7	55.2	52.9	52.0	59.5	33.1
11	Foretomach	62.4	−371.5	43.1	51.8	77.5	−132.1
	Entire gut	82.6	−100.5	92.7	63.9	91.6	65.4

TABLE 5

Ratio of disappearance of each digestible component in the forestomach to that of the entire gut

Trial No.	Organic matter	Crude protein	Ether-extract	Crude fiber	NFE	Crude ash
	(%)					
1	80.0	11.7	2.6	97.4	81.2	−19.0
2	81.8	52.1	73.2	97.7	92.9	−46.0
3	61.7	18.5	−2.9	101.6	65.7	−53.9
4	70.8	19.0	2.4	97.3	81.4	−76.8
5	85.4	−0.4	61.9	99.4	96.9	−2.2
6	75.2	−20.0	66.3	93.0	84.3	13.4
7	66.2	13.1	4.6	100.2	77.2	−61.0
8	73.4	39.8	28.8	102.4	84.4	−40.4
9	72.1	−2.3	25.6	96.3	82.5	−85.7
10	82.3	40.9	18.8	104.6	90.1	−42.7
11	75.5	−369.6	46.5	81.0	84.6	−202.1

amount of saliva secreted paralleled the intake of hay. The results of Trial 11 permit the calculation of the nitrogen content of saliva (13.65 mg. of nitrogen was contained in 100 ml. of saliva). Based on this value, the true digestibility of protein in the forestomach was calculated, with the results shown in Table 6. It is found that 52.6–87.0% of digestible protein of feed was absorbed from the forestomach.

DISCUSSION

Initially, the authors were concerned that some difference might result in the digestibility values obtained from the forestomach-separated goats and those from untreated goats. Such apprehensions occurred for the following reasons:

TABLE 6

Amount of saliva secreted and the true digestibility of protein in the forestomach in each trial

Trial No.	Water taken[a]		Saliva[a] secreted	True digestibility of protein in the forestomach	True digestibility of protein in the forestomach/digestible protein in feed
	Water drunk	Water contained in feed			
	(ml.)	*(g.)*	*(g.)*	*(%)*	*(%)*
1	938	78	3,521	32.9	66.0
2	.....	3,586	2,094	46.5	62.9
3	423	53	2,957	30.6	56.8
4	747	59	2,974	29.5	52.6
5	86	41	3,430	42.0	87.0
6	321	46	3,722	32.7	79.9
7	305	38	2,494	37.8	64.1
8	640	64	3,473	41.3	66.6
9	226	52	3,359	27.7	60.0
10	283	58	3,662	46.5	84.2
11	106	16	2,073	.....	.....

[a] Average per day.

(a) Since the digestive tract was incised between the omasum and abomasum, the content of the gut was inevitably exposed to the air midway in digestion. (b) In untreated goats, the digesta moves through the digestive tract continuously, but in the operated goats comparatively large amounts of digesta were poured into the abomasum hourly. (c) Moreover, the activity of the experimental animal was limited rigidly. A supplementary experiment (9) was conducted to determine whether there were any differences in digestibility between the untreated and the operated goats. As a control for Trial 2, an untreated goat was fed the same green rye as in Trial 2. The results were: digestibility coefficient of organic matter 23.8%, crude protein 77.1%, ether extract 63.4%, crude fiber 74.9%, NFE 72.5%, and crude ash 56.4%. As a control for Trial 4, the same feed was given to an untreated goat. The results were: digestibility coefficient of organic matter 64.4%, crude protein 62.5%, ether-extract 61.6%, crude fiber 51.6%, NFE 69.3%, and crude ash 29.7%. These results show there was little difference between the figures obtained with the operated animal and the untreated one (*cf.* Table 4). Accordingly, it must be concluded that the extent of digestion in the operated goats was essentially unchanged from that of untreated goats.

Concerning the true digestibility of protein, the range of absorption of dietary protein from the forestomach was about 52 to 87%. Since ammonia and urea are the principal nitrogen compounds absorbed from the rumen (24), these results indicate that relatively large amounts of protein nitrogen were absorbed from the rumen as ammonia, and that a large proportion of protein was probably converted into microbial protein (13, 14). On the other hand, the apparent digestibility coefficients of protein in the forestomachs were proportional to the protein levels in the feed. In other words, at the lower protein levels it became negative, and at the higher levels it became positive. These facts seem to indicate that at the higher protein levels the nitrogen compounds in feed were partly

absorbed from the rumen as well as the nitrogen secreted in saliva; whereas, at low protein levels no feed protein was absorbed and salivary nitrogen was synthesized into protein. However, the nitrogen of both feed and saliva must be considered as one unit in the rumen.

As previously shown by McNaught and others (16, 17), the conversion rate of ammonia into microbial protein was affected by the presence of carbohydrate and by its quality and quantity. Since the protein level varied in each of these trials, it is impossible to assess the effect of carbohydrate on protein synthesis in the rumen. However, in rations containing small amounts of readily available carbohydrate (Trials 5, 6, and 10), the true digestibility of protein in the forestomachs was about 80% of the digestive protein (Table 6). A large amount of ether-extractable material was absorbed from the forestomach when only roughage was fed; whereas, a small, or negative, amount of it was absorbed when hay and concentrates were fed. This can be explained by the fact that ether-extractable material was formed from carbohydrate and protein by rumen bacteria, as indicated by the work of Hale *et al.* (6, 8). It was reported that the nature of the dietary fat was different from that of the fat in the rumen (20, 21). This indicates that a large portion of the ether-extractable materials are digested and absorbed from the rumen, and that new forms are synthesized.

In these trials, the digestible crude fiber almost completely disappeared while in the rumen, but in previous experiments this was not so and, in certain instances, the largest part of it was digested in the hind gut (1, 6, 8). Perhaps such conclusions were erroneous because the ratio technique was employed.

The disappearance of digestible NFE from the forestomach ranged from 65.7 to 96.9%. NFE is chiefly changed into volatile fatty acids and absorbed from the rumen, but the remaining 3 to 34% of the digestible NFE was, perhaps, absorbed from the small intestine as glucose. A study of the difference between volatile fatty acids and glucose as energy sources for ruminants is an interesting problem.

The amount of saliva secreted and its ash content were so large that the digestibility of crude ash in the forestomach showed negative values except in Trial 6. Since potassium, chloride, sodium, phosphate, etc. are absorbed from the rumen (4, 18, 22, 23), some of the minerals in the feed must have been partly absorbed from the rumen.

Assuming that there was no water secreted into and absorbed from the forestomach, the amount of saliva secreted was estimated as indicated previously and the results are shown in Table 6. Raynard *et al.* (19) reported that water was absorbed from the omasum and Tsuda (24) suggested the possibility of absorption of water from the rumen. The value given in Table 6 for the volume of saliva is actually an estimate of the increased amount of water that occurs during digestion in the forestomach. Since the value obtained in Table 6 approximates the values estimated for salivary volume of sheep (3, 15), this would indicate that there was only a small amount of water exchange occurring in the forestomach.

At the determination of endogenous nitrogen, neither the water exchange in the forestomach nor the variation of the nitrogen contents of saliva was considered. Therefore, the values of true digestibility of protein in the forestomach were only estimated ones. These results of estimations are presented here, in the hope of contributing to the process of clarification of the problem of rumen nitrogen digestion.

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EFFECT OF RECURRING PREGNANCY ON MAMMARY GLAND GROWTH IN MICE[1]

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SUMMARY

It was shown by the increase of total desoxyribosenucleic acid (DNA) that mammary gland growth continues during the latter half of pregnancy. This observation was rather unexpected, since morphological study of lobule-alveolar growth can not distinguish between additional cell growth and the initiation of milk secretion. These data indicate for the first time that cell multiplication continues throughout pregnancy.

The effect of recurring pregnancy on mammary gland growth was studied, employing albino mice bred successively without nursing periods. Maximum growth of the mammary glands was attained in triparous animals. A slight decline in total growth was observed in quadriparous animals. Their age at this time was estimated to be equivalent to the age of cows which begin to decline in yearly milk and fat yields. It was also confirmed that increased mammary gland growth in multiparous animals was more closely related to hormonal stimuli associated with recurring pregnancy and lactation than to increasing body weight. Primiparous mice after 3 mo. of involution had mammary glands containing DNA equivalent to mice pregnant for six days. No variation in average litter size was observed during four consecutive pregnancies.

Milk and fat secretion in dairy cattle has been shown to increase with advancing age up to about 8 yr. The age-production relation has been used extensively to formulate age-correction factors for converting milk and fat production records to their mature equivalent. Two obvious factors are involved in the increase in milk secretion: (a) the increase in body weight up to 8 yr. (7, 9) and (b) recurring pregnancy and lactation. In the analysis of these data it was suggested that recurring pregnancy (and factors associated with pregnancy and lactation) influenced the increase in lactation with age far more than did the increase in body weight. Confirmation of this relation has been provided by an analysis of milk records of Swedish dairy cattle (5). It was shown that milk yield per lactation depends not only on age up to maturity but also on the number of lactations. To reach maximum yield the cows must, on the average, have had at least three pregnancies.

The most obvious factor in recurring pregnancy is growth of the mammary gland. While it has been inferred during the last 30 yr. that additional growth of the mammary gland was stimulated with each pregnancy up to the time of maximum milk yield, no quantitative method of determination of mammary gland growth has been available to prove that additional growth occurred.

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During the past few years, a quantitative method of determining the growth of the mammary gland has been developed (2, 6, 13). It has been shown that the desoxyribosenucleic acid (DNA) content of each mammary gland cell is constant during pregnancy (3). Thus, as the cells multiply, the amount of DNA in the glands increases. A chemical method of increased sensitivity for determination of DNA recently has been described (11). With this method available, it seemed desirable to determine *(1)* the extent of gland growth (as measured by total DNA) during stages of the first pregnancy, and *(2)* whether gland growth was extended by two, three, or four successive pregnancies.

EXPERIMENTAL PROCEDURE

Albino mice fed a standard laboratory feed were bred when weighing about 28–30 g. Pregnancy was dated from the morning a vaginal plug was observed. The multiparous mice were remated immediately after parturition, following the removal of the young. Not all mice conceived immediately, so there was a variable period before the second and successive pregnancies were initiated. At the time indicated in Table 1, the animals were sacrificed, skinned, and three-fourths of the mammary glands of each mouse taken for DNA determination. The remaining one-fourth of the glands was prepared for whole-mount examination, to observe the type of gland development.

The gland tissue for DNA determination was frozen, the fat extracted with hot alcohol and ether and ground to a fine powder. Then, 30 mg. of the gland was extracted twice with 5 ml. of hot 5% trichloroacetic acid to remove the DNA, which was then determined by the Webb-Levy method (11).

Experimental observations. As controls, a group of unmated mice with extensive duct development were used. The average total DNA of the group was 1,704 μg. (Table 1). A certain number of mice, when sacrificed at six and 12 days after coitus, were found not to be pregnant. As coitus stimulates the maintenance of the corpora lutea, i.e., pseudopregnancy (8), the mammary glands of these animals were included in the study. It will be noted that gland growth occurred (DNA, 2,269 μg. per animal) at six days, but was not further increased at 12 days. Since this value is based upon a limited number of animals, the DNA may be increased as a more representative sample is obtained. In the first pregnancy, progressive growth of the mammary gland was observed at six, 12, and 18 days, as indicated by increasing DNA content. The marked increase in DNA from the 12th to the 18th day was unexpected since, on the basis of the morphological observations, it had been assumed that the major part of lobule-alveolar growth occurred during the first half of pregnancy. It was observed, also, that growth of the glands of pregnant animals was greater than that of pseudopregnant animals at the same stages. While further study will be required to confirm these observations, they suggest the possibility that the pregnant state (fetal membranes?) contribute hormones which cause greater mammary gland growth than the ovarian hormones (corpora lutea of pseudopregnancy) alone.

Considering the extent of mammary gland growth produced at the end of the first pregnancy (mean total DNA) as a base, it will be noted during the second pregnancy that there was 28.70% additional growth of mammary gland tissue

TABLE 1

Effect of recurring pregnancy on the mammary gland growth in mice

Reproductive state	Stage of gestation	No. of mice	D.F.F.T.[a] Wt. (mg.) Mean ± S.E.		D.F.F.T. Wt. Wet wt. of glands (%) Mean ± S.E.		DNA (μg.) mg. D.F.F.T. Mean ± S.E.		Total DNA (μg.) Mean ± S.E.		Initial body wt. (g.) Mean ± S.E.		Total DNA (μg.) Initial body wt. (g.) Mean ± S.E.	
Virgin		7	44.0	2.5	4.0	0.7	38.8	3.5	1,704	180	30.8[b]	0.7	54.4[c]	5.1
Nullipara	6 Days after sterile coitus	15	52.5	2.1	5.7	0.4	42.9	0.5	2,269	126	31.6	0.8	71.5	3.8
	12 Days after sterile coitus	5	54.8	3.1	4.3	0.8	39.8	1.4	2,202	159	32.8	1.1	77.8	8.2
Primipara	6 Days of pregnancy	10	63.2	2.4	6.9	0.5	46.3	2.0	2,853	153	31.4	1.0	90.4	4.9
	12 Days of pregnancy	10	80.1	4.6	6.3	0.3	51.4	2.8	4,118	319	32.8	1.0	125.3	9.1
	18 Days of pregnancy	10	121.8	8.6	8.4	0.4	49.4	1.6	5,965	353	32.0	1.3	185.8	7.0
Bipara	18 Days of pregnancy	10	154.0	5.9	9.8	0.5	49.3	1.9	7,677	438	33.4	1.2	235.6	20.2
Tripara	18 Days of pregnancy	10	165.9	10.9	9.3	0.3	45.4	0.8	7,730	497	36.1	1.4	215.0	9.2
Quadripara	18 Days of pregnancy	10	146.0	8.6	9.2	0.2	46.5	1.2	6,982	366	36.9	0.9	188.5	6.3
Primipara	Sterile over 3 mo. after first parturition	7	69.3	8.0	6.5	0.9	40.2	3.9	2,720	286	32.2[b]	1.7	83.8[c]	7.9

[a] D.F.F.T. = Dry, fat-free tissue.
[b] Final body weight.
[c] Total DNA/final body weight.

(total DNA) and during the third pregnancy an additional further slight increase. Thus, the total additional mammary gland growth induced by two successive periods of pregnancy amounted to an increase of 29.59% over that induced by the first pregnancy. To the extent that additional epithelial cells present represent additional capacity to secrete milk by the tripara mice, it is assumed that their lactational capacity would thus be increased by about 30% as a result of two additional pregnancies. Since the average body weight of the mice at the beginning of the first pregnancy was 32.0 g. and of the tripara mice 36.1 g., the additional mammary gland growth induced was only slightly related to the increasing body weight. In other words, there is a marked increase in total DNA per gram increase in body weight in the bipara mice. It will be noted, also, that the weight ratio of dry fat-free tissue to wet tissue of the multiparous mice was greater than that of the primiparous mice. With only slightly higher nutritional maintenance requirement of the bipara and tripara animals, the increased lactational capacity of the animals with larger mammary glands would increase the nutritional efficiency for milk production of the latter groups.

The group of quadripara mice showed a 9.68% decline in average total DNA, in comparison with the tripara group. The decline in capacity for mammary gland growth is believed to represent a decline in capacity for the secretion of the hormones essential (directly and indirectly) for mammary gland growth. This compares with the gradual decline in yearly milk and fat production of dairy cattle after peak production is reached at 7 or 8 yr. Brody (1) reported that 1 mo. in the mouse was equivalent to 15.3 mo. in the cow. The quadripara mice in this experiment were in range of 5.5–6.5 mo. of age, averaging 6 mo. at the end of their pregnancies. This is equivalent to cows 85 to 100 mo. of age (see Brody's Figure 19.7). Thus, the quadripara mice were of an age comparable to cattle which begin to decline in yearly milk yield. It has been known that thyroid function has a relation to the age, and that features of the hypothyroid state are similar to some of the features of senescence. Recently, it has been reported that the thyroxine secretion rate of rats decreases with advancing age (12). Unpublished preliminary data also showed that thyroxine secretion rate per unit of body weight in aged mice is lower than in younger animals by radioactive replacement technique (10). Therefore, the decline of mammary gland growth in quadripara mice may be due to a decline of thyroxine secretion rate as well as pituitary activity and other physiological functions. In primipara mice which were not bred again for 3 mo. or more, there was observed involution of the mammary glands to an extent comparable to that observed at the sixth day of pregnancy. Even in animals at 25 days after parturition, without recurring pregnancy and nursing, there was marked involution of mammary alveolar tissue (not included in Table 1).

Ingram *et al.* (4) has recently shown that age of mice does not influence litter size up to the fourth; however, a decline in litter size occurs thereafter. The results of the present experiment show no direct relationship between the litter size and mammary gland growth, since average litter size in all groups was constant, ranging from 9.0 to 9.5.

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EFFECTS OF VARIOUS AMOUNTS OF CHLORTETRACYCLINE IN THE RATIONS OF LACTATING DAIRY CATTLE

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SUMMARY

Aureomycin[1] (chlortetracycline) was fed to lactating dairy cattle at levels of 0, 0.1, 0.5, and 1.0 mg. per pound of body weight per cow per day for a period of 2 wk. Although two of the four cows in each of the two groups receiving the higher doses showed slightly depressed appetites shortly after the trial was started, feed consumption returned to normal after a brief period of adjustment. During this adjustment period, milk production also was reduced, but generally returned to similar relative levels of the controls within a few days. No marked clinical effects were noted in the group receiving 0.1 mg. per pound of body weight, and milk from these cows was negative for the presence of the antibiotic. Slight amounts of chlortetracycline were found in the milk of cows on higher feeding levels and these slight amounts were eliminated from all cows' milk by 48 hr. after the feeding of the antibiotic had been discontinued.

Early work by Bell *et al.* (2) in beef cattle and Colby *et al.* (3) in sheep appeared to support the theory that oral administration of antibiotics to ruminating cattle or sheep would be harmful, since bacteria are essential to the digestive mechanism of these animals. Later workers (1, 5, 8, and 15) reported that Aureomycin (chlortetracycline) could be fed without harmful effect to ruminating cattle at levels in excess of the total dose commonly fed to calves. These later reports renewed interest in the application of antibiotics for the feeding of ruminating animals, and much work with beef cattle and sheep has been reported in the literature in the last few years. The acceptance of antibiotics in fattening rations for beef cattle and sheep is now widespread and is a common, although not universal, practice in commercial operations.

Loosli and Warner (11), Rusoff and Haq (14), and Jacobson *et al.* (9) reported that levels of 130–700 mg. of chlortetracycline per cow per day had no harmful effect on feed consumption or milk production. However, relatively little interest was shown in adapting the results of beef cattle work to dairy cattle, since the major result—improved weight gains—is not of primary importance in commercial dairy herds. Also, it was not known what effect the feeding of antibiotics would have on milk intended for cheese manufacturing, although Loosli and Warner (11) and Martin *et al.* (12) reported that feeding relatively high levels of chlortetracycline to dairy cattle did not interfere with the use of milk for the manufacture of cheese.

Public health officials were concerned about the possible presence of antibiotics in the milk and the effect they might have on persons consuming the milk. Henderson *et al.* (7) studied this question and their work indicated that the feeding of 0.1 mg. of chlortetracycline per pound of body weight per cow per day did not produce measurable amounts of antibiotic either in milk or in the blood. This

[1] The trademark of the American Cyanamid Company for the antibiotic chlortetracycline is Aureomycin.

is the dose which has been permitted for commercial use by the Food and Drug Administration. The object of our investigation was to repeat the work of Henderson *et al.* (7), who fed several levels of chlortetracycline, to provide additional information on this subject for federal and state authorities who wished to see the work repeated. The major emphasis in this report will be placed on the levels of antibiotic found in the milk from cows fed three different levels of chlortetracycline.

EXPERIMENTAL PROCEDURE

Sixteen normal lactating dairy cows were allotted to one of four treatment groups, with four cows being allotted to each group. Aureomycin (chlortetracycline) Crumbles, containing 2.0 g. of the antibiotic per pound, was added to the rations of the cows in each group, at levels to provide the following daily doses of antibiotic:

Group 1—Control. No chlortetracycline.

Group 2—0.1 mg. chlortetracycline per pound of body weight per cow per day.

Group 3—0.5 mg. chlortetracycline per pound of body weight per cow per day.

Group 4—1.0 mg. chlortetracycline per pound of body weight per cow per day.

The antibiotic was placed in the concentrate portion of the ration, with one-half the dose fed at each of the two daily feedings. The antibiotic feeding was conducted for a period of 2 wk.

Observations on total feed consumption were made during this period. Total pounds of concentrate and hay consumed each day and the speed at which they were consumed were recorded. Total consumption of feed revealed the effect, if any, on appetite; whereas, this factor plus speed of consumption, especially of the concentrate, indicated whether palatability of the ration was affected.

The effect on milk production and any evidence of toxicity or digestive disturbances caused by the trial procedure were recorded. Milk samples were obtained from each cow at the start of the experiment. Samples also were taken from each of the treated cows at 24-hr. intervals, continuing for three days after the feeding of the antibiotic was stopped. Antibiotic levels in the milk were determined by a modification of the Food and Drug Administration cylinder-plate technique (4), which has a sensitivity level of 0.01 γ/ml.

RESULTS

On the second day of antibiotic feeding, two cows on each of the 0.5 and 1.0 mg. feeding levels refused part of the grain ration. On the third day, one of these cows in each group was still off-feed to some degree. The feeding procedure was rechecked and it was found that these—as well as the other cows in the same groups—were receiving 1.0 and 2.0 mg. per pound of body weight, respectively. The dose was, therefore, readjusted to that planned in the original design

of the experiment. All animals quickly returned to full feed consumption thereafter; no apparent effect on palatability of the feed was noted.

Cow 777 became ill during the experiment. Since this animal was not eating grain or roughage, its milk production was not included and the milk was negative for the antibiotic. The clinical symptoms were those of acetonemia, and finding acetone in the urine and milk confirmed this diagnosis. This condition did not appear to be a result of the treatment, since no ill effects from feeding antibiotic have been reported by other workers (1, 5, 6, 8, 9, 11, 12, 14, 15) who fed both lower and higher, as well as similar, doses of chlortetracycline.

No signs of toxicity or other untoward effects directly attributable to the antibiotic were observed, other than the temporary reduction in feed intake referred to earlier and the accompanying transitory reduction in milk production.

Figure 1 shows the average daily milk production for each group from four days before antibiotic feeding through four days after it was eliminated from the ration. Milk production in all groups dropped on the third day after chlortetracycline feeding started. The experiment was conducted in the summer and the weather was extremely hot on this day. However, while the drop in the group receiving 0.1 mg. of chlortetracycline per pound of body weight per cow per day was similar to that of the controls, the lowered production in the other groups was of a greater magnitude and was apparently related to the administration of the antibiotic.

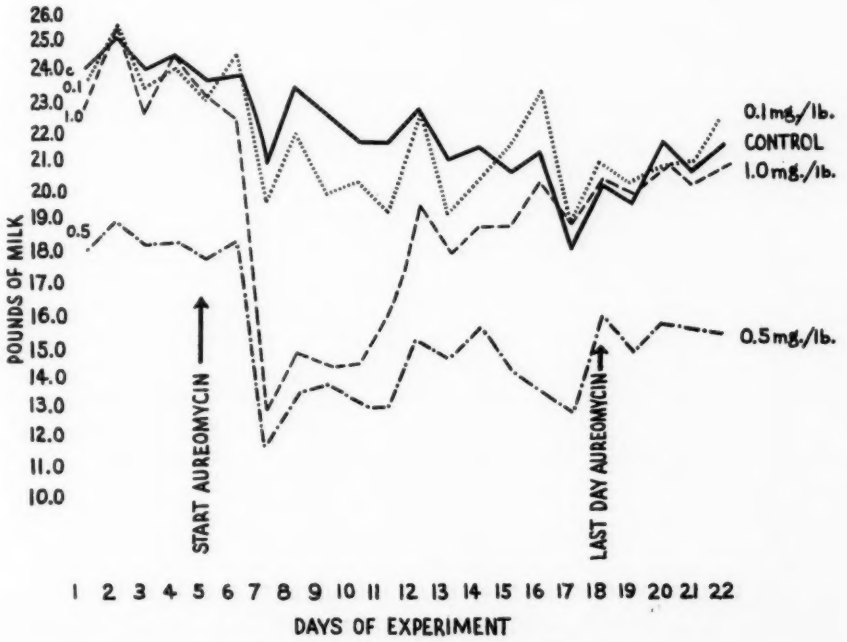

FIG. 1. Average daily milk production.

In the group receiving 1.0 mg. of chlortetracycline per pound of body weight per day, production returned within a few days to a level comparable to that of the control and the low level groups. The cows receiving 0.5 mg. chlortetracycline per pound per day increased in production gradually, but did not come up to the same production as other cattle until some time after the experiment was concluded. Their initial production was below that of the other cows and they returned to producing comparable levels in only a slightly longer time than the others.

The levels of chlortetracycline in the milk are shown in Table 1. Milk from the controls and all of the treated cows was negative at the start of the experiment and is, therefore, not included in the table. Some of the early readings in the 0.5 and 1.0 mg. treatment groups were high because of the higher actual dosage, but later readings are considered more typical and are in agreement with the work of Henderson (6). All milk was negative 24 hr. after the removal of the antibiotic from the diet, with the exception of samples from two cows on each of the high levels of treatment; their milk was negative after 48 hr.

DISCUSSION

The transitory depression in feed intake reported is similar to that occasionally seen for beef cattle shortly after the antibiotic is introduced in the ration. In all probability, this reflects an adjustment of the rumen flora. This was not

TABLE 1

Amounts of chlortetracycline in milk during 14 days of continuous feeding

Sampling time in hours after start of medication	Feed treatment[a]							
	0.5 mg. chlortetracycline/lb body weight/day				1.0 mg. chlortetracycline/lb body weight/day			
	Cow No.				Cow No.			
	772	776	777[b]	778	764	765	766	767
	(μg/ml milk)							
0	0	0	0	0	0	0	0	0
24	0.03	0.02	0.03	0.01	0.04	0.06	0.04	0.05
48	0.10	0.05	0.03	0.04	0.13	0.20	0.11	0.14
72	0.11	0.21	0.03	0.05	0.17	0.23	0.20	0.13
96	0.05	0.05	0.03	0.04	0.14	0.16	0.20	0.05
120	0.03	0.03	0.01	0.02	0.03	0.13	0.17	0.04
144	0.06	0.05	0	0.04	0.08	0.10	0.21	0.04
168	0.07	0.05	0	0.03	0.08	0.11	0.14	0.05
192	0.07	0.06	0	0.03	0.06	0.08	0.07	0.06
216	0.07	0.05	0	0.02	0.07	0.07	0.08	0.05
240	0.04	0.02	0	0.02	0.06	0.04	0.11	0.12
264	0.06	0.06	0	0.02	0.06	0.09	0.13	0.06
288	0.09	0.03	0	0.02	0.07	0.05	0.06	0.08
312	0.08	0.06	0	0.03	0.08	0.06	0.06	0.09
336[d]	0.06	0.05	0	0.02	0.05	0.04	0.06	0.08
360	0.04	0.03	0	0	0	0	0.03	0.06
384	0	0	0	0	0	0	0	0
408	0	0	0	0	0	0	0	0

[a] Milk from control cows and cows receiving 0.1 mg. chlortetracycline/lb body weight/day negative for chlortetracycline.

[b] Cow 777 removed from treatment on fifth day because of illness.

[c] Method sensitivity, 0.01 γ/ml.

[d] Chlortetracycline removed from the feed after the 14th day.

seen in all herds nor in all animals within a herd where gross evidence of this adjustment was apparent. The higher doses which were given early in the trial are not believed to have been responsible per se for the effect, because even higher doses have been fed to cattle with no apparent effect, and the same doses used for spot treatment in the experiments of Henderson (6) had no untoward results.

The feeding of 0.1 mg. of chlortetracycline per pound of body weight per cow per day produced no effect on feed consumption and only a very slight decrease in milk production, which was of short duration. This effect on production was not reported by Henderson *et al.* (7) and was reported only occasionally by others (10, 13) using chlortetracycline at this level in commercial dairy herds. It would appear that, as in beef cattle, ten times the recommended level is safe to feed but that even the recommended level may occasionally cause digestive upsets that are overcome within a few days.

Milk production paralleled feed consumption. Since the cows receiving 1.0 mg. of chlortetracycline per pound of body weight returned to normal production relatively quickly, failure of the group on the intermediate dosage schedule to return as quickly to full production was attributed to individual idiosyncrasy. All cows, including the controls, were producing less at the end of the experiment than at the beginning, probably because of the unusual weather conditions.

Milk samples were negative for chlortetracycline in the cows fed 0.1 mg. per pound of body weight per day. These results are in agreement with the work of Henderson *et al.* (7), cited earlier. Other workers who fed similar dosage levels of antibiotic did not report assay of the milk; therefore, no direct comparisons can be made with other studies.

The most common range of levels of antibiotic in the milk for the group fed 0.5 mg. per pound of body weight after correction of the dosage error was 0.02 γ/ml to 0.06 γ/ml, and this increased in direct proportion to the dosage at the higher treatment level. Chlortetracycline, fed at 0.5 mg. per pound of body weight, therefore produced only minimal levels in the milk. Loosli and Warner (11) reported no antibiotic in the milk when 700 mg. of chlortetracycline was fed daily for ten days. This is slightly more than 0.5 mg. per pound of body weight. Since the assay method used was not described, it is not known whether the technique was less sensitive or whether this was a question of individual idiosyncrasy. Henderson *et al.* (7) reported detectable amounts of antibiotic in the milk (without giving exact figures) at the higher dosage levels. Recent communication (6) indicates that the levels reported here are similar to those obtained in the earlier work (7).

Since Martin *et al.* (12) indicated that 0.32 mg. per pound of body weight did not show measurable amounts of chlortetracycline in the milk, 0.5 mg. per pound of body weight approaches the lowest feeding level that will show activity in the milk. As previously indicated, the Food and Drug Administration's approved feeding level is 0.1 mg. per pound of body weight per cow per day. This dose is safe for cattle and, because approximately five times the dose is required to produce a detectable amount of antibiotic in the milk, it is unlikely to affect the suitability of milk for cheese manufacture or consumption by sensitive individuals.

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FREEZING BOVINE SEMEN. V. PRACTICABILITY OF COLLECTING AND FREEZING A LARGE NUMBER OF SUCCESSIVE EJACULATES [1, 2]

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SUMMARY

From six to 37 successive ejaculates were collected during 12, 2-hr. depletion trials with eight dairy bulls, and used to investigate (a) the proportion of ejaculates acceptable for freezing and (b) the freezability of the acceptable ejaculates. Only ejaculates showing an initial motility of 30% or more and a minimum of 150 million motile spermatozoa were frozen. On this basis, the percentage of culled ejaculates increased as the number of consecutively collected ejaculates increased. Considering the first five consecutive ejaculates, only 6.7% were culled; among the first ten consecutive ejaculates, 11.6% were culled; whereas, among all 257 ejaculates collected, 35.8% were culled. There was no significant difference in the freezability of the acceptable ejaculates among the first five or first ten individual ejaculates, and freezability was comparable with that obtained with ejaculates collected from the same bulls when on a frequency of two or six ejaculates per week. Beyond the tenth ejaculate, freezability was generally, but not significantly, poorer and collection of more than ten consecutive ejaculates probably would be of no practical value. Pooling acceptable ejaculates from a depletion trial resulted in freezability comparable to freezing the ejaculates separately. With adequate sexual preparation, the collection of six or seven consecutive ejaculates from a given bull should make available large numbers of sperm at one time, which should freeze satisfactorily.

Preservation of bull spermatozoa by freezing has become a practical procedure in routine artificial breeding. In most associations, bulls are ejaculated once or twice every four to seven days. Thus, semen from these bulls must be frozen frequently in relatively small quantities. A considerable saving in both time and labor would result if bulls could be ejaculated a number of times consecutively at weekly or monthly intervals to provide larger quantities of semen for freezing. This would permit the establishment of a large bank of frozen semen from a particular bull very rapidly.

Several reports have shown that a greater percentage of spermatozoa survive freezing to −79° C. in second than in first ejaculates when they are collected consecutively (4, 6, 9, 10). Willett and Ohms (10) attributed the superiority in freezability of sperm from the second over the first ejaculate to unknown characteristics of the cells and not to differences in seminal plasma. VanDemark *et al.* (9) postulated that the maturity of the sperm might be a factor affecting freezability. Holt (3) and Madden (6) suggested that the higher concentration of sperm in the first ejaculate than in the second or third ejaculate may play an important role in contributing to poorer freezability of sperm in the first

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ejaculate. Willett and Ohms (10) and Kinney and VanDemark (4) reported progressive improvement in the freezability of consecutively collected ejaculates through the fourth, with a gradual decrease thereafter through the 18th to 20th, ejaculate.

This paper reports the results of a study to determine (a) the proportion of ejaculates acceptable for freezing when a large number of ejaculates were collected during 2-hr. depletion trials and (b) the freezability of the acceptable ejaculates.

EXPERIMENTAL PROCEDURE

Eight bulls of the Guernsey, Holstein, and Jersey breeds were allowed to ejaculate without restraint, false mounting, or encouragement during a 2-hr. depletion trial (1). Four of the bulls were subjected to a second 2-hr. depletion trial seven days following the first, so that a total of 12 different collection days (depletion trials) was evaluated (Table 1). A total of 257 ejaculates was obtained, or an average of 22 ejaculates per depletion trial (range six to 37).

Only those ejaculates having an initial motility of 30% or more and a minimum of 150 million motile spermatozoa were frozen. Each of the 165 ejaculates which met the above requirements was diluted as soon as possible after collection to 30 million motile sperm per milliliter in 5 ml. of heated fresh skimmilk and cooled to 5° C. in 2 to 3 hr. An equal volume of heated skimmilk containing 26% glycerol by volume and 2.5% fructose by weight was added to the diluted semen

TABLE 1

A comparison of the post-thawing motility immediately after freezing of ejaculates collected during depletion trials with that of ejaculates collected at a frequency of two or six ejaculates per week

Breed and bull	Age of bull	No. of ejaculates collected during the 2-hr. depletion	All ejaculates frozen		Ejaculates 1 through 10 frozen		Two or six ejaculates per week	
			No. of ejaculates	Av. % motility	No. of ejaculates	Av. % motility	No. of ejaculates	Av. % motility
	(yr.)							
G-1	2	7	6	42.5	6	42.5	4	35.0
G-2	2	9	8	52.8	8	52.8	12	45.6
G-3	7	11	10	56.5	10	56.5	11	50.7
G-3[a]		11	11	51.1	10	50.2		
Total and av.		38	35	51.6	34	51.4	27	46.1
H-1	4	6	5	44.5	5	44.5	14	50.9
H-2	2	29	13	34.8	6	40.4	2	40.0
H-3	5	30	28	37.8	10	41.0	9	46.9
H-3[a]		37	17	34.6	10	39.5		
Total and av.		102	63	36.9	31	40.9	25	48.6
J-1	6	37	18	25.8	4	21.4	7	28.9
J-1[a]		20	13	47.9	10	48.0		
J-2	6	31	21	44.3	10	41.8	10	37.5
J-2[a]		29	15	41.0	10	52.5		
Total and av.		117	67	39.3	34	42.1	17	34.0
Grand total and av.		257	165	41.0	99	44.8	69	44.0

[a] Bulls appearing twice were subjected to two depletion trials at a seven-day interval.

at 5° C. in five equal portions at 6-min. intervals. The final diluted semen contained 1,000 units each of penicillin and dihydrostreptomycin and 15×10^6 motile sperm per milliliter. During a 6-hr. equilibration period, 1.0-ml. portions of diluted semen were placed in 1.6-ml. glass ampules and sealed. The samples were frozen in an ethyl alcohol bath by gradual addition of dry ice to lower the temperature of the bath at the rate of 3° C. per minute from +5 to −15° C., 10° C. per minute from −15 to −50° C., and approximately 12 to 15° C. per minute from −50 to −79° C. In five of the depletion trials the ejaculates also were pooled and samples of the pooled semen were processed and frozen as above.

One of each pair of duplicate ampules was thawed in a 5° C. water bath immediately after freezing, while the other was thawed after ten days of storage at −79° C. Duplicate estimations of the percentages of motile sperm were made on each ampule and averaged for statistical analysis. The ampules were coded and randomly selected for thawing to reduce observer bias.

RESULTS AND DISCUSSION

Based on Table 1, 92, or 36%, of the 257 ejaculates were not acceptable for freezing. Fourteen of the 92 ejaculates had insufficient numbers of motile sperm, while 78 had less than 30% motile spermatozoa. Of the ejaculates with less than 30% motile spermatozoa, 62, or 80%, exhibited no motility. This is similar to the observation of Almquist and Hale (1), who noted that, except for an increase in the incidence of samples with no motility, the per cent of motile sperm was not appreciably altered by successive ejaculations. Considering only the first ten consecutive ejaculates (total of 112) collected in the present experiment, 13, or 12%, were undesirable for freezing. Of the first five consecutive ejaculates collected (total of 60) only four, or 6.7%, were not suitable for freezing.

Table 1 compares the average freezability of acceptable ejaculates in the 12 depletion trials with the average of those for the first ten consecutive ejaculates and the average of the two to 14 first or second ejaculates collected from the same bulls when they were on a collection frequency of two or six ejaculates per week (two ejaculates one day per week −2 ×; two ejaculates each on Monday, Wednesday, and Friday −6 ×). The differences among the three groups of ejaculates and among breeds were small. The average freezability of the ejaculates in the 1 to 10 category was slightly higher than the average of the ejaculates collected on frequencies of two or six per week, whereas the average for all samples collected and frozen in the 2-hr. depletions was slightly lower. The average freezability of ejaculates from the Holstein bulls was lower than that of the ejaculates collected at a frequency of two or six per week. The reverse was true for the Guernsey and Jersey bulls. However, final conclusions on breed differences can not be made because of the very small number of bulls in this study.

As shown in Table 1, the average freezability of acceptable ejaculates collected from three of the four bulls 1 wk. following a previous depletion trial was lower than in the first trial. Considering only the first ten ejaculates collected, however, this relationship was true for only two of the four bulls. Nevertheless, this indicates that for some bulls it may be desirable to allow more than 1 wk.

of rest between depletion trials for optimum freezability when ten or more consecutive ejaculates are collected.

The average semen characteristics for all the ejaculates frozen from the eight depletion trials at weekly intervals and from the first ten consecutive ejaculates in all depletion trials, as well as for those collected at a frequency of two or six ejaculates per week are presented (Table 2). With the four bulls subjected to two depletion trials, the average number of sperm per ejaculate in each trial was the same. Semen volume per ejaculate was lower in the second trial, but was offset by an increase in concentration. There was no effect on percentage of motile sperm. These data do not explain the decreased freezability of the ejaculates in the second depletion trial. The average volume and concentration of the acceptable ejaculates among the first ten consecutive ejaculates in all 12 depletion trials was significantly lower than for ejaculates collected at frequencies of two or six weekly. The pattern of decline in semen characteristics is similar to that presented by Almquist and Hale (1).

Table 3 summarizes in increments of five the freezability of the 165 acceptable ejaculates and the percentage of the 257 consecutively collected ejaculates which were suitable for freezing. There was a progressive decline both in the proportion of suitable ejaculates and in the freezability among ejaculate groups. The largest decrease in freezability occurred between the 11-15 and 16-20 ejaculate groups. The decrease in post-thawing motility accompanied the decrease in the percentage of motile sperm prior to freezing. These results agree with those of VanDemark *et al.* (9). In the present experiment, the average percentage of motile sperm for the ejaculates frozen from the first ten consecutive ejaculates (total of 99 ejaculates frozen) when thawed immediately after freezing was 44.8%; whereas, the average for those frozen from ejaculates 11 through 37 (total of 66 ejaculates

TABLE 2

Comparison of semen characteristics for all ejaculates frozen from depletion trials at weekly intervals and of ejaculates frozen from the first ten consecutive ejaculates in 12 depletion trials with those collected at a frequency of two or six ejaculates per week

Acceptable ejaculates from two successive depletion trials at a seven-day interval from four bulls

	1st depletion trial	2nd depletion trial	Difference
Total number of ejaculates collected	109	97	12
Total number of ejaculates frozen	77	56	21
Volume of semen per ejaculate *(ml.)*	3.31	2.33	0.98
Motile sperm *(%)*	59.9	60.5	0.6
Number of motile sperm per milliliter semen *($\times 10^6$)*	535	802	267
Number of sperm per ejaculate *($\times 10^6$)*	1,833	1,806	27

Acceptable ejaculates from the first ten collected in 12 depletion trials

	Depletion trials	2 or 6 ejaculates per week	Difference
Total number of ejaculates collected	112	.....	.....
Total number of ejaculates frozen	99	69	.....
Volume of semen per ejaculate *(ml.)*	3.36	4.09	0.73[a]
Motile sperm *(%)*	62.0	64.3	2.3
Number of sperm per milliter semen *($\times 10^6$)*	887	1,398	521[b]

[a] $P < 0.05$.
[b] $P < 0.01$.

TABLE 3

Comparison of the acceptability and freezability of ejaculates collected during a 2-hr. depletion trial

(Weighted average for 12 collections from eight bulls)

Ejaculate group	No. of ejaculates collected	No. of ejaculates frozen	Per cent of ejaculates frozen	Pre-freezing motility	Post-thawing motility after storage at −79° C. for		
					0 day	10 days	Av.
				(%)	(%)	(%)	(%)
1–5	60	56	93.3	56.5	45.4	37.0	41.2
6–10	52	43	82.7	56.0	44.1	33.7	39.0
11–15	37	24	64.9	54.8	38.5	30.9	34.7
16–20	35	18	51.4	49.2	34.6	21.2	28.1
21–25	30	15	50.0	51.0	32.0	19.5	25.9
26 and over	43	9	20.9	52.2	33.3	20.0	27.1
Total and av.	257	165	64.2	54.6	41.0	31.2	36.1

frozen) was only 38.3%. In addition, 54.5% of ejaculates 11 through 37 (total of 145 ejaculates collected) were unsatisfactory for freezing, whereas only 11.6% of ejaculates 1 through 10 were not satisfactory. Therefore, it appears that it would not be desirable to collect more than ten consecutive ejaculates from dairy bulls for freezing and storage.

Since the previous results indicated that it probably would be undesirable to collect more than ten consecutive ejaculates from a bull for freezing, the data were analyzed on the basis of the first five and the first ten consecutive ejaculates.

TABLE 4

Freezability of five and ten ejaculates collected in succession from dairy bulls

Ejaculate number	Prefreezing motility	Post-thawing motility after storage at −79° C. for		
		0 day	10 days	Av.
	(%)	(%)	(%)	(%)
	Average of 11 depletion trials from eight bulls[a]			
1	59.1	42.0	31.4	36.7
2	54.1	48.0	39.8	43.9
3	60.9	48.0	40.4	44.2
4	60.4	48.0	40.4	44.2
5	59.1	50.9	42.5	46.7
Av.	58.7	47.4	38.9	43.1
	Average of seven depletion trials from four bulls[a]			
1	59.3	45.4	32.5	38.9
2	52.9	48.2	36.4	42.3
3	62.9	50.3	40.0	45.2
4	60.0	50.0	37.5	43.8
5	57.9	50.7	38.9	44.8
6	58.6	48.2	32.5	40.4
7	59.3	50.7	37.9	44.3
8	57.1	44.6	34.3	39.5
9	55.0	42.5	34.3	38.4
10	60.7	41.1	30.0	35.5
Av.	58.4	47.2	35.4	41.3

[a] Includes only depletion trials in which the first five or the first ten ejaculates were acceptable for freezing.

No ejaculates were discarded. Data presented in Table 4 show the freezability of the first five ejaculates for 11 trials and of the first ten for seven of the 12 trials. The remaining trials were excluded because one or more ejaculates did not meet the minimum standards or ten ejaculates were not obtained. In both analyses of variance no significant differences among individual ejaculates were found when tested with the respective highly significant bull × ejaculate interaction. Although there was a highly significant ($P < 0.01$) decrease in viable sperm after ten days of storage at −79° C., the interaction between ejaculates and length of storage at −79° C. was not significant. The interaction between bulls and loss of viable sperm during storage at −79° C. was highly significant ($P < 0.01$) and this agrees with other reports (4, 6, 7). The freezability of consecutive ejaculates increased through the fifth to seventh ejaculate, with a slight decrease thereafter through the tenth ejaculate. However, the decrease was not of sufficient magnitude to warrant culling any of the first ten ejaculates.

Table 4 also shows a difference in freezability in favor of all but the ninth and tenth ejaculates over the first ejaculate. The superiority of the second ejaculate over the first agrees with other studies (4, 9, 10). In the present experiment, the second ejaculate froze better ($P < 0.05$) and exhibited less loss of motile sperm after ten days of storage at −79° C. ($P < 0.01$) than did the first. Although perhaps influenced by the lack of special sexual preparation prior to collection, the second ejaculate was higher in volume, fructose content, and initial motility, but was lower in sperm concentration ($P < 0.05$), total sperm, and total motile sperm. There was no difference in rate of motility or per cent of live sperm as determined by differential staining.

Table 5 compares the freezability of the pooled ejaculates from five of the depletion trials with the average freezability of the same ejaculates individually. In general, post-thawing motility of the pooled semen was slightly, but not significantly, higher than the average of the individual ejaculates. Pooling consecutive ejaculates would greatly simplify the processing of semen for freezing.

Based on the average ejaculate characteristics shown in Table 2, a total of

TABLE 5

Effect of pooling acceptable ejaculates on the post-thawing motility of spermatozoa

Bull	Prefreezing motility			Post-thawing motility after storage at 79° C.					
				Av. of individual ejaculates			Pooled ejaculates		
	No. of ejaculates frozen	Av. of individual ejaculates	Pooled ejaculates	0 day	10 days	Av.	0 day	10 days	Av.
		(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
G–3[a]	11	61.4	65.0	51.1	31.8	41.5	57.5	42.5	50.0
J–1	18	37.8	40.0	25.8	15.4	20.6	32.5	17.5	25.0
J–1[a]	13	58.8	50.0	47.9	40.0	43.9	45.0	45.0	45.0
J–2	21	55.0	50.0	44.3	39.5	41.9	40.0	30.0	35.0
J–2[a]	15	51.7	55.0	41.0	33.3	37.2	52.5	50.0	51.2
Total and av.	78	52.9	52.0	42.0	32.0	37.0	45.5	37.0	41.2

[a] These trials were conducted seven days after a previous depletion trial.

1,232 ampules of semen containing 15×10^6 motile sperm per ampule could be frozen from ten consecutive ejaculates. Only 490 ampules could be frozen on a collection frequency of two ejaculates a week. Thus, approximately 2.5 times as many ampules of semen could be frozen at weekly intervals by collecting ten consecutive ejaculates a week, rather than two ejaculates a week. In addition, the higher frequency of ejaculation should save time in processing and freezing semen. Other studies at this laboratory (2) show that with more intensive sexual preparation than used in the present study, larger sperm numbers can be collected at frequencies of two or six ejaculates per week. With adequate preparation for each ejaculate, the majority of sperm available for ejaculation should be obtained by collecting six or seven ejaculates rather than ten or more at weekly intervals without preparation. Fertility trials are needed to substantiate the generally favorable laboratory results.

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INFLUENCE OF PROLONGED INCUBATION OF BULL SEMEN AT 37° C. ON FRUCTOLYSIS, LACTIC ACID, AND MOTILITY [1]

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SUMMARY

Eighteen semen samples, three from each of six dairy bulls, were concentrated by centrifuging and removing supernatant plasma to one-half of the original volume. The sperm and remaining plasma were extended 1:6 with 2.9% Na-citrate and divided into 20 tubes of 1 ml. and incubated at 37° C. Duplicate tubes were removed at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, and 6.0 hr. of incubation, for determination of pH, fructose, lactic acid, and motility rating on a 0 to 10 scale (10 = 100%). At 0 hr. of incubation the averages were: (a) pH—6.87; (b) fructose—4.27 ± 0.33 mg/ml of semen; (c) lactic acid—0.84 ± 0.10 mg/ml of semen, and (d) motility—6.56 ± 0.89. pH remained essentially unchanged during incubation. Fructose utilization by 10^8 sperm/ml/hr was 2.02 ± 0.18 mg. during the first 0.5 hr. of incubation. Motility ceased when fructose concentration was less than 0.06 mg/ml of extended semen and was seriously impaired at observed levels of 0.14 to 0.29 mg/ml. Lactic acid accumulation reached levels of 0.6 mg/ml of extended semen, but was not a principal cause of reduced motility. It is recommended that fructose levels in metabolism studies be expressed as mg/ml of extended semen, as well as the concentration per milliliter of whole semen, since different extension rates are frequently used.

Lardy and Phillips (12) recognized that glycolysis was the primary source of energy for motility of bull sperm. Following the identification of fructose as the principal sugar in semen (13) many studies, as earlier reviewed (3, 5, 6), have shown that bull sperm metabolize fructose at variable rates, depending on motility, sperm cell concentration, extent of dilution, incubation time and temperature, and amount of fructose initially present in the incubate.

Hopwood *et al.* (11) first recognized that removal of fructose by sperm fitted, in an empirical way, a unimolecular (first-order) reaction. These workers presented a method for expressing fructolysis rates independent of sperm concentration. Later, Mixner *et al.* (14) also showed that fructose utilization rate by incubating sperm followed essentially the first-order reaction, and derived a method for expressing fructolysis rate independently of initial levels of fructose present. Freund *et al.* (10) investigated the problem further and concluded that, for comparative purposes, fructolysis rates should be expressed independently of sperm concentration and levels of fructose initially present.

Lactic acid accumulates in an incubating sperm-substrate mixture when a sugar is metabolized. However, sperm are capable of using lactic acid further; under a variety of experimental conditions from 9 to 75% of the fructose loss has not been accounted for as lactic acid. Like fructolysis rate, lactic acid gain during incubation shows a highly significant correlation with initial motility and

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motility at the end of the incubation period (9). Fructolysis rates by 10^9 sperm/ml and lactic acid gain (5, 6, 9) have been positively correlated with non-return rates on a between-bull basis. High lactic acid accumulations have been positively associated with maintenance of motility under conditions of incubation (6, 9) and storage (16).

Maintenance of motility during semen storage at 5° C. remains the single most practical test of fertilizing capacity. Thus, it appeared important to examine the relationship between sperm motility and incubating substrate levels of fructose and lactic acid. It was the purpose of this experiment to investigate the substrate levels of fructose required to support progressive motility. Lactic acid accumulation was also followed, to determine if lactic acid is metabolized at a greater rate when substrate fructose is very low.

EXPERIMENTAL PROCEDURE

Eighteen semen samples were collected from six mature bulls in the State College of Washington dairy herd. Three samples, pooling first and second ejaculates, were collected from each bull at approximately weekly intervals. Immediately after collection, 6 ml. of semen was placed into a 50-ml. pyrex conical tube and centrifuged at 750 g for 8 min. After centrifugation, 3 ml. of supernatant plasma was removed to increase the sperm : plasma ratio so that seminal fructose would be more completely utilized by 6 hr. of incubation. This procedure was believed satisfactory because Albright *et al.* (1) have shown that centrifuged twice-washed sperm, resuspended in milk, maintain motility as well as centrifuged sperm resuspended in the original plasma. Motility regained immediately following centrifuging was essentially the same as before. The separated sperm and remaining plasma were extended 1 : 6 with 2.9% Na-citrate containing 1 mg. of streptomycin/ml of extended semen. The extender was forcibly ejected into the compressed sperm cells. One milliliter of the semen-extender mixture was placed into 15-ml. centrifuge tubes for incubation in a water bath at 37° C. Subsequent samplings were in duplicate for determination of motility, fructose, and lactic acid. Samplings were made at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, and 6.0 hr. of incubation. Immediately after determining motility, 5 ml. of cold (5° C.) 10% trichloroacetic acid were added to duplicate tubes after each interval of time, then thoroughly mixed and placed in an ice water bath. After approximately 30 min., the samples were centrifuged, filtered, and stored in a refrigerator for fructose and lactic acid determinations the following day.

Motility was subjectively rated on the basis of progressively motile sperm on a 0 to 10 scale where 10 was estimated as 100%. Low-power microscopy was used. Sperm concentrations were determined in duplicate with a hemocytometer. Fructose was measured by the method of Roe (15), as modified and used by Erb *et al.* (5). Lactic acid was determined by the method of Barker and Summerson (2), as modified by Ehlers and Erb (3). The colorimetric determinations for fructose and lactic acid were with an Evelyn colorimeter. The pH was determined with a Beckman glass-electrode pH meter. Statistical procedures were as outlined by Snedecor (17).

RESULTS AND DISCUSSION

The 18 extended semen samples at the start of incubation averaged as follows: (a) Motility—6.56 ± 0.89; (b) 10^9 sperm/ml—2.11 ± 0.14; (c) lactic acid—0.84 ± 0.10 mg/ml, and (d) fructose—4.27 ± 0.33 mg/ml. Figure 1 shows that fructose use by 10^9 sperm/ml/hr declined from an acceptable rate of 2.02 ± 0.18 mg. during the first 30 min. of incubation to below a 0.5 mg. rate between 1.0 and 1.5 hr. Motility averaged less than 1.0 by 3 hr. of incubation. Lactic acid reached near maximum concentration by 1.5 hr. of incubation and this coincided with less than 1.0 mg. of fructose per milliliter of semen (only 0.14 mg/ml of incubate). The pH declined very little, but the rate of decline was greatest during the early part of incubation. An extra tube containing approximately 1 ml. of extended semen was available for each sample and was incubated along with the original 20. After motility ceased, and in some cases 1 to 2 hr. later, addition of one or two drops of a fructose solution or 0.5 ml. of seminal plasma to these nonmotile samples resulted in revived motility. The degree of revival was, in general, related to time after motility ceased. If fructose or seminal plasma was added soon after no progressive motility was observed, sperm movement increased to approximately that observed at the start of incubation. This is interpreted to

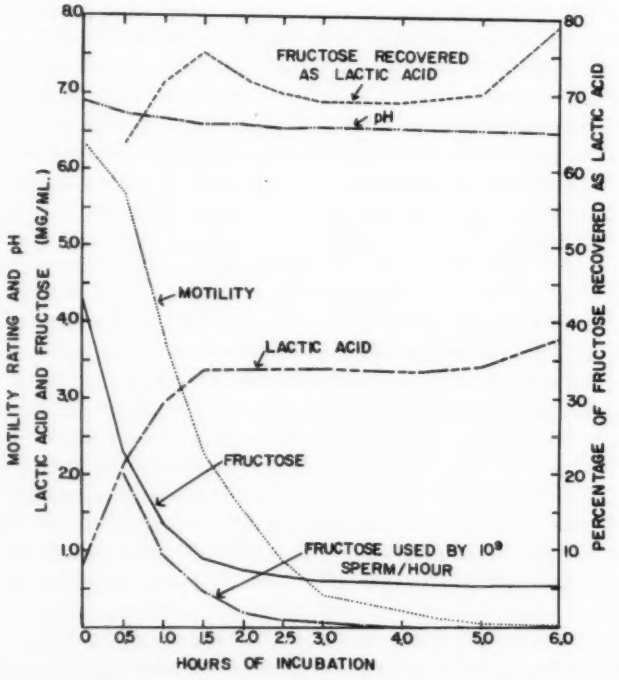

FIG. 1. Changes in motility, fructose, lactic acid, fructose utilization by 10^9 sperm/hr, fructose recovered as lactic acid, and pH during 6 hr. of incubation at 37° C.

mean that under the conditions of this study lactic acid accumulation was not great enough to reduce motility.

Erb *et al.* (5) estimated that fructose in excess of 1 mg/ml of semen was necessary to maintain a reasonable utilization rate by 10^9 sperm. Because the extension rate was 1 : 4, the fructose level per milliliter of incubate was one-fifth as high, or 0.2 mg/ml of incubate. In this study, the semen was extended 1 : 6. Figure 1 shows 1.35 mg/ml of fructose after 1 hr. and the concentration per milliliter of incubate was one-seventh, or 0.19 mg/ml. The motility at this time averaged 3.7 as compared with 6.6 for the earlier study (6). The data in Figure 1 indicate that fructose concentrations of approximately 0.15 mg/ml of incubate (one-seventh the values in Figure 1, to compensate for extension) are at the critical level, as evidenced by the rapid decline in progressive motility and the small increases in lactic acid. The correlation, using covariance, between fructose (mg/ml) and motility was 0.68 for all samples in this study and 0.66 for samples within time of incubation and bull. The correlation between fructose use/hour and motility was 0.85 for all samples and 0.41 for samples within time of incubation and bull. This latter value corresponds with a sample within-bull correlation of 0.38 in an earlier study (6).

In 16 of the 18 samples, the available fructose was nearly all used before motility rated less than two. The remaining two semen samples were from different bulls. The fructose initially present was, respectively, 4.1 and 5.7 mg/ml of semen and the utilization rate/hour was above average for the first hour. Both samples were below average in sperm concentration. Motility was maintained longer than average. Lactic acid accumulation proceeded normally. Since stress caused by low fructose was not created in these two samples, they were omitted from the summary shown in Figure 2.

The data for 16 samples exhausting fructose in 6 hr. or less of incubation were summarized by motility, independently of time of incubation. Concentrations of fructose and lactic acid are shown in Figure 2 on a basis of mg/ml of extended semen, instead of the usual mg/ml of semen (Figure 1). Figure 2 shows that lactic acid concentrations were maximum (0.61 mg/ml) for motility ratings of 2.

There is little possibility that concentrations at this level inhibit motility because in a previous study (9) motility after incubation for 1 hr. was highest for samples exceeding 0.6 mg. of lactic acid/milliliter of semen. Figure 2 also shows that motility essentially ceases when fructose concentration is less than 0.06 to 0.08 mg/ml of extended incubating semen. These values were adjusted for sperm cell volume (6, 8); thus, the range of 0.06 to 0.08 mg/ml is higher than if adjustment had not been made.

From Figure 1 it can be calculated that motility declined 10% and fructose declined 45% during the first 0.5 hr. of incubation. Fructose decreased to 0.34 mg/ml of extended semen at this time. Motility declined 42% and fructose 68% during the first 1 hr. of incubation, and fructose decreased to 0.18 mg/ml of extended semen. This suggests that the level of fructose required for good motility is between 0.34 and 0.18 mg/ml of extended semen. From Figure 2 it

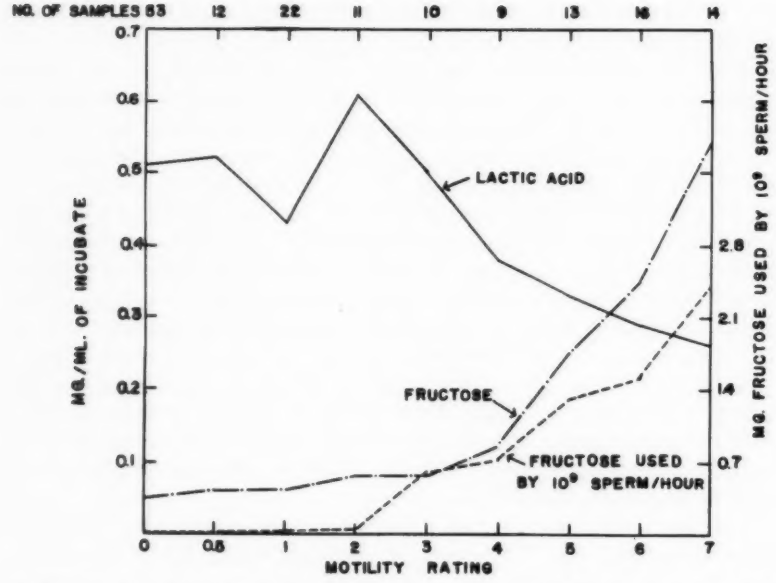

FIG. 2. The relationship between motility rating and lactic acid, fructose, and fructose utilization by 10^9 sperm/ml/hr.

appears that approximately 0.25 mg. of fructose per milliliter of incubating semen-extender mixture is required to prevent excessive decline in average motility. Since the semen samples varied in initial motility, a motility rating of five would represent a sizeable drop in one sample and none in another. A clearer picture was obtained by determining the average fructose at the incubation time preceding a drop of at least one in motility rating and comparing with average fructose at the time a drop of this magnitude was observed. Fructose declined 50% or to an average of 0.29 mg/ml of extended semen before a noticeable decline in motility (average of 6%) occurred. During the next 0.5 hr., motility rating was 4.8, a decline of 22%, and fructose was down an additional 27%, or to an average of 0.14 mg/ml of extended semen. Figure 2 describes the changes quite well for motility ratings of less than five. Thus, the critical level of fructose for good motility under the conditions of this experiment was less than 0.29 mg/ml of extended semen and more than 0.14 mg/ml and probably not far from 0.25 mg/ml. This coincides with 1.25 mg/ml of semen when extended 1:4 prior to incubation (5) and 1.75 mg/ml when extended 1:6 (Figure 1).

General discussion. Ehlers and Erb (3) have observed that whole semen–extender volume relationships (phosphate) are of greater importance than actual sperm concentration. Fructose utilization and lactic acid production were similar through an extension range of 1:1 to 1:8, but declined at 1:16 and 1:24. Based on the fructose initially present, the 1:8 extension left an average of 0.68 mg.

of fructose per milliliter of incubate and a 1:16 extension averaged 0.32 mg/ml (3). The utilization of fructose by 10^9 sperm for the first hour of incubation was 1.4 mg. The motility averaged 6.5 (3). This corresponds with 0.34 mg/ml of fructose and 1.4 mg/hr for a motility rating of six in Figure 2. Milk has been shown to provide energy for maintenance of motility (1). Therefore, it is significant that when Ehlers and Erb (3) extended semen 1:24 with milk, lactic acid production was slightly greater than when extended at 1:4. Purposely creating conditions to insure exhaustion of fructose failed to show that an appreciable extra amount of lactic acid was used. Instead, motility declined rapidly when fructose in the incubating semen was below approximately 0.1 mg/ml.

This study shows that fructose in the absence of other glycolyzable sugars is necessary for maintenance of motility and that 0.14 to 0.29 mg/ml of incubate is required to maintain average motility. Additional fructose or seminal plasma revived motility in semen incubates after initial rates had declined to zero. This shows that lactic acid levels (maximum reached was 0.6 mg/ml of incubate) were not high enough to inhibit motility. Also, energy to maintain the living sperm cell may be entirely from nonglycolytic sources (12), because motility was markedly revived even after 1-2 hr. in the nonmotile state at 37° C. The revival of motility to approximately initial levels was probably higher than would have been the case if no seminal plasma had been removed. In reality, slightly over one-half of the fructose initially present was removed in the seminal plasma, because the sperm cells contain little or no fructose. Under these conditions, none of the intracellular reserves, and probably very little of the substances normally bound to the peripheral surfaces of the sperm cell, were removed. Therefore, the ratio of intra- and extra-cellular nutrient supply was altered considerably. The results imply that the well-being of nonmotile sperm is not impaired if sufficient energy from nonglycolyzable sources is present.

Progressive motility as a practical test of semen quality serves indirectly as a measure of metabolic activity initially and during storage (6). More consideration should be given to expressing fructose concentration in mg/ml of extended semen, rather than on an amount in the unextended semen. Little or no progressive motility can be expected when fructose is below 0.06 mg/ml of extended semen.

This study is not in disagreement with principles set forth concerning the relationship between level of initial fructose and sperm concentration and the fructose utilization rate during incubation (5, 10, 11, 14). Consideration of motility would assist in distinguishing between sperm (semen samples) incapable of utilizing the sugar present and those capable of further sugar use when an exogenous source of energy is supplied in the extender.

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A STUDY OF BOVINE FOLLICULAR FLUID AND ITS EFFECT ON OXYGEN CONSUMPTION BY BOVINE SPERMATOZOA[1]

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SUMMARY

Five semen samples were obtained from each of two bulls and used to determine the effects of follicular fluid on oxygen consumption by washed spermatozoa. The spermatozoa in follicular fluid utilized 4.6 times more oxygen than did an equal number of spermatozoa in saline. It was found that 36.6% of this increase was due to glucose, 26.1% to protein; and the remaining 37.3% was apparently due to other unidentified, heat-stable, nondialyzable substances.

The pH of follicular fluid increased from about 7.4 to about 8.9 during incubation in Warburg flasks for 3 hr. at 37° C. This rise in pH was found to be due to the diffusion of carbon dioxide out of the fluid and its absorption by KOH in the center wells of the flasks. Fresh follicular fluid contained about 0.498 g. of carbon dioxide per liter.

Follicular fluid is thought to function in the nourishment, protection, and transportation of ova while they are being ovulated and carried into the oviduct. If this is true, it is very likely that spermatozoa also come into contact with follicular fluid at or near the time of fertilization. It has been reported that follicular fluid exerts a stimulating action on sperm motility (1,4) fructolysis and glycolysis (2, 3), and on respiration (3). Lutwak-Mann (2) concluded that the effect of follicular fluid on sperm fructolysis was due to its glucose content. However, preliminary work by Olds and VanDemark (3) indicated that the stimulating action of follicular fluid on sperm respiration was due to nondialyzable substances.

The present study was undertaken in an effort to learn more about the nature of bovine follicular fluid and how it affects the metabolic activity of bovine spermatozoa.

METHODS

Follicular fluid was collected from ovarian follicles of cows being slaughtered by a local meat-packing company. Composite samples of the fluid were stored at −10° C. until used. The following preparations of follicular fluid were made and used as diluents for washed spermatozoa: (1) Unaltered follicular fluid, (2) follicular fluid which had been dialyzed against water to remove salts and small molecules and then against physiological saline to reconstitute normal osmotic pressure, (3) follicular fluid which had been deproteinized by saturation with ammonium sulfate; the supernatant was dialyzed against water to remove the ammonium sulfate and then against saline to adjust the osmotic pressure; the protein precipitate was resuspended to the original volume and then dialyzed against water followed by physiological saline, (4) follicular fluid was heated to 55° C. for 30 min., for the purpose of inactivating enzymes.

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[1] The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

Five ejaculates were collected from each of two Jersey bulls at weekly intervals. The spermatozoa were washed three times in physiological saline (total of four centrifugations) and adjusted to a concentration of 1×10^9 cells per milliliter.

Oxygen uptake was determined by placing 0.2 ml. of the sperm suspension and 0.8 ml. of diluent in a Warburg flask and incubating for 3 hr. at 37° C. At the end of the incubation period, samples were removed from each flask for determination of pH and for microscopic examination.

Results and Discussion

The average oxygen uptake by bovine spermatozoa in the various media is shown in Figure 1. It may be observed that oxygen uptakes over the 3-hr. period were very similar for spermatozoa in whole follicular fluid, heated follicular fluid, and in dialyzed follicular fluid to which glucose had been added. There were no statistically significant differences among these three fluids. However, dialyzed follicular fluid produced significantly lower oxygen uptakes than any of the three aforementioned fluids. Likewise, there was no significant difference between uptakes produced by the solution containing follicular fluid protein and the dialyzed supernatant after protein precipitation. However, spermatozoa in both of these fluids produced significantly higher uptakes than did the spermatozoa in the physiological saline control.

These observations suggest that glucose metabolism accounts for a sizable portion of the oxygen consumption by spermatozoa in follicular fluid. However, the data also suggest that follicular fluid protein contributes to the oxygen consumption in some way other than through its enzyme content. Since the dialyzed supernatant fluid remaining after protein precipitation also produced higher uptakes than the saline control, it appears that follicular fluid contains a heat-

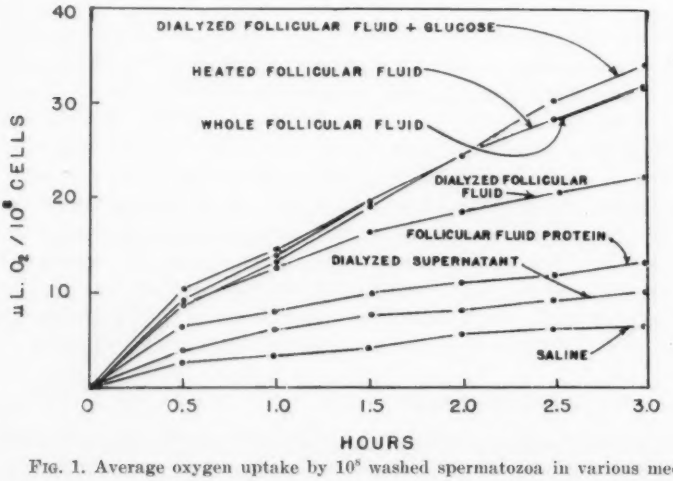

Fig. 1. Average oxygen uptake by 10^8 washed spermatozoa in various media.

stable nonprotein substance of large molecular size which contributes to oxygen uptake by spermatozoa. One might expect the effects of follicular fluid protein and of the dialyzed supernatant to add up to the effect of dialyzed whole follicular fluid. Actually, the sum of these two fell somewhat short of accounting for the effects of dialyzed follicular fluid. Therefore, it appears that ammonium sulfate treatment may have had an adverse effect which persisted on some follicular fluid component even after removal of the ammonium sulfate by dialysis. These data are summarized in Table 1.

Examination of Table 1 reveals that glucose is apparently the primary component of follicular fluid which is removed by dialysis, and it accounts for about 37% of the oxygen consumption by spermatozoa in whole follicular fluid. The protein of follicular fluid appears to account for about 26% of the oxygen consumption in whole follicular fluid. The remaining 37% appears to be attributable to a heat-stable, nonprotein, nondialyzable substance and also to a substance whose effectiveness is destroyed by the action of ammonium sulfate.

TABLE 1

Average oxygen uptake by 2×10^8 washed spermatozoa from ten samples of semen during three hours' incubation at 37° C. in saline and in various preparations of follicular fluid

Diluting media	Oxygen uptake	Increase over saline
	(*µl.*)	(*µl.*)
Unaltered follicular fluid	63.2	49.6
Heated follicular fluid	64.0	50.4
Dialyzed follicular fluid + glucose	69.9	56.3
Dialyzed follicular fluid	45.1	31.5
Saline solution of follicular fluid protein	26.5	12.9
Supernatant of f.f. after protein precipitation	23.3	9.7
Saline	13.6	0.0

The pH of the various fluids was determined before and after the 3-hr. incubation periods. While all of the fluids were near neutrality at the beginning of incubation, it was observed that whole follicular fluid samples (whether heated or unheated) consistently acquired pH's of about 8.85 during the incubation period; whereas, all of the samples prepared by treatments involving dialysis had an average pH of about 6.9 following incubation. At first, it was thought that perhaps the increase in alkalinity in whole follicular fluid was due to the metabolic activity of spermatozoa. However, incubation of whole follicular fluid without spermatozoa revealed that the pH rose to about 9.3 after 3 hr. in the Warburg flask. It was then concluded that the increase in alkalinity might be due to an escape of CO_2 from the solution. This idea was further substantiated when it was found that alkalinity did not increase when KOH was left out of the center well (KOH absorbs CO_2). Finally, the amount of CO_2 which could be driven off from follicular fluid by acid treatment was estimated with the Warburg apparatus. It was found that follicular fluid contained about 0.498 g. of CO_2 per liter. Calculations indicated that this amount, if allowed to escape from follicular fluid, could very likely explain the pH changes.

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EFFECTS OF GLYCEROL AND FRUCTOSE ON LIVABILITY, MOTILITY, AND ANAEROBIC GAS PRODUCTION OF BOVINE SPERMATOZOA[1]

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SUMMARY

In vitro manometric determinations of gas production by bovine spermatozoa under anaerobic conditions indicated a slight but statistically nonsignificant inhibitory effect of glycerol. The inclusion of 1% fructose with 1.0, 3.3, or 10.0% glycerol caused an immediate and marked stimulation of gas production by the spermatozoa.

Glycerol plus fructose maintained a greater degree of motility and livability of bovine spermatozoa at the end of 105 min. of incubation at 38° C. than did either fructose or glycerol alone. Under the conditions of this study, 1% glycerol plus 1% fructose was the apparent optimum for maintenance of spermatozoan motility and livability.

In 1949, Polge *et al.* (23) discovered that glycerol, a trihydroxy alcohol, would protect fowl spermatozoa during freezing and thawing. Since then, the mode of action (11, 12, 14, 25) and rate of entry (10, 13) of glycerol have been studied for several types of tissues. Although the practical aspects of freezing bovine spermatozoa have received considerable attention, relatively little work has been done on possible localization and mode of action of glycerol in these cells. Proof of entry has been presented largely by metabolic studies. Mann and White (16) found that glycerol was utilized aerobically but not anaerobically by ram spermatozoa. O'Dell *et al.* (21) observed that glycerol-1-C^{14} was utilized anaerobically by bovine spermatozoa and converted, in part, to radioactive carbon dioxide. White *et al.* (27) found that glycerol, under the conditions of their study, increased the oxygen consumption of bovine spermatozoa. Pickett and Merilan (22) employed an autoradiographic technique in an attempt to determine the loci of glycerol action and found glycerol-1-C^{14} to be associated with all parts of the spermatozoa. However, more of the C^{14} appeared to be with the nuclear area of the head than with the other parts.

The investigations of Mann (15) have shown fructose to be the normal glycolyzable carbohydrate present in bovine semen and that fructose is broken down, in part at least, to lactic acid. It has been further found that the rate of glycolysis is greater in nitrogen than in air (2, 3, 15). This study was conducted in an attempt to provide additional information concerning the metabolism of various concentrations of glycerol and glycerol-fructose combinations under anaerobic conditions.

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Materials and Methods

Bull semen was collected by means of an artificial vagina from five bulls maintained as part of the University of Missouri Agricultural Experiment Station Dairy Herd. Semen motility was rated according to the method of Herman and Madden (8) and only samples of four motility or better were used in the experiments. The spermatozoa were washed in calcium-free Krebs-Ringer phosphate (KRP), prepared according to Umbreit *et al.* (26), by suspending the semen in 167.0% of its original volume, centrifuging for 10 min. at 600 g, and removing the same amount of material that was added. Sperm cell concentration for each washed suspension was determined by the hemocytometer method (8).

Anaerobic metabolism of the spermatozoa was measured by gas production from the substrate, with KRP as the buffer. The measurements were made at 38° C. in a rectangular constant-volume Warburg Respirometer accommodating 14 reaction vessels, and adjusted to a shaking speed of 114 strokes per minute. To each of the flasks was added 2.0 ml. KRP, 0.5 ml. of the washed sperm suspension, and 0.5 ml. of the substrate (glycerol, fructose, or glycerol-fructose) was placed in the side arm. Final volume in each flask was 3.0 ml. The flasks were gassed with a mixture of 95.0% nitrogen and 5.0% carbon dioxide, using a manifold gassing arrangement.

After placing the flasks in the 38° C. water bath, they were allowed to equilibrate for 10 min. Following equilibration, readings were taken every 5 min. for 30 min., to establish a base. The side arms were then tipped and readings taken at 5-min. intervals for 75 min.

At the end of the experimental run, motility and per cent alive were determined on each flask. The per cent alive was calculated as the average of duplicate determinations made according to the method proposed by Mayer *et al.* (17).

Results and Discussion

The data presented in Figure 1 indicate that gas production by bovine spermatozoa was slightly inhibited (not statistically significant at 5% level) by all concentrations of glycerol studied. The control flasks contained some residual fructose, since the washing procedure used in these studies was not designed to remove all the fructose present in the seminal plasma but merely to dilute it while keeping the spermatozoan concentration high. Therefore, the controls in this study probably utilized the remaining fructose, whereas in the flasks containing glycerol, the glycerol in some way interfered with fructose utilization. However, the inhibition was not directly proportional to the final concentration of glycerol present. In the flasks containing 8.3 and 10.0% glycerol, most of the inhibition occurred during the first 15–30 min. after tipping; whereas, at lower glycerol concentrations, less inhibition occurred in the same time interval. This indicated that perhaps glycerol concentrations of 7–10% should be added stepwise, as done by some investigators (1, 5, 6, 20, 24).

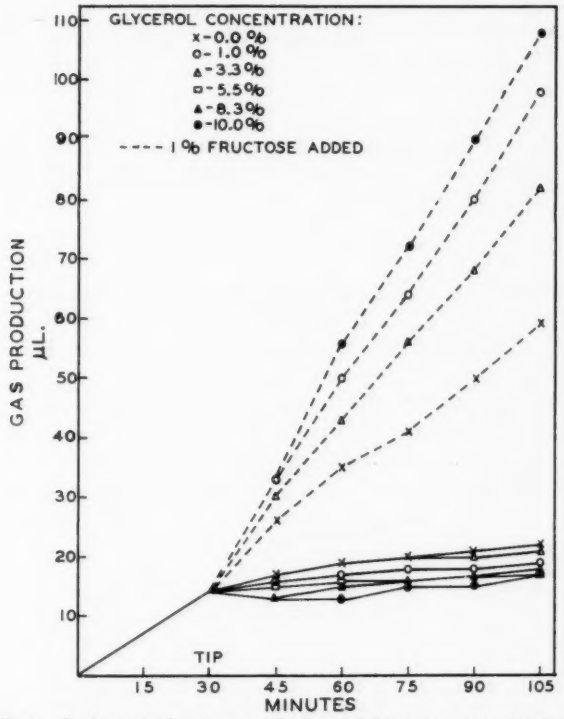

FIG. 1. Effect of glycerol, fructose, and glycerol-fructose concentrations on anaerobic metabolism of bovine spermatozoa (1.57×10^7 cells/ml) suspended in calcium-free Krebs-Ringer phosphate. Average for split-sample determinations with 15 individual semen ejaculates.

The addition of 1.0% fructose to the flasks, at the same time 1.0, 3.3, and 10.0% glycerol were added, caused an immediate and marked stimulation of gas production. However, these results do not show whether the increased gas production was the result of the utilization of fructose or glycerol, or both. In view of some recent investigations, it appears probable that the carbon dioxide was being produced from both glycerol and fructose. Mann (15) and Moore (19) have shown that glucose and fructose appear to be utilized through essentially identical pathways. Flipse and Almquist (4) have shown that lactic acid produced from glucose-C^{14} by bovine spermatozoa was, in part, broken down to carbon dioxide. O'Dell *et al.* (21) found that glycerol-1-C^{14} was utilized by bovine spermatozoa under anaerobic conditions and converted in measurable amounts to radioactive carbon dioxide. An alternate possibility is that fructose might accelerate the entry of glycerol into the cell, as has been postulated by White *et al.* (27) for arabinose, a nonglycolyzable pentose.

After 105 min. of incubation, motility and per cent alive determinations were made on each flask shown in Figure 1. The data taken from this study

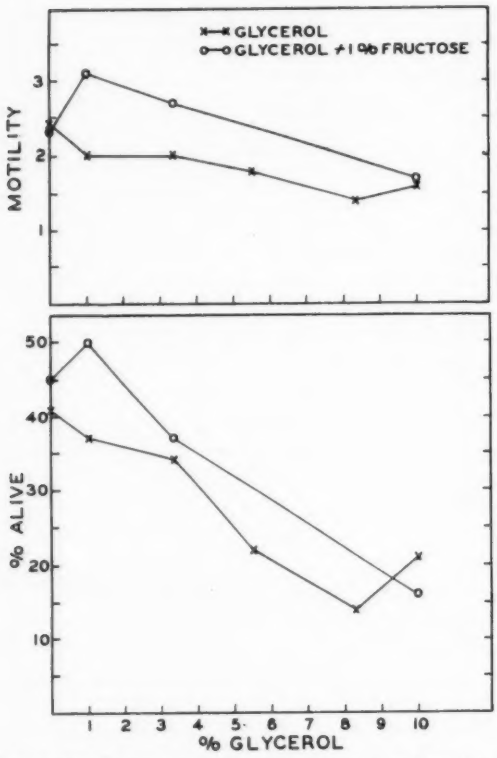

FIG. 2. Effect of various concentrations of glycerol and fructose (± glycerol) on spermatozoan motility and survival after 105 min. incubation at 38° C.

are presented in Figure 2. Mixner and Saroff (18) reported that glycerol levels above 4% in diluters containing egg yolk, skimmilk, or whole milk interfered markedly with the dead-alive stain; however, that effect was not noted in preliminary trials for these studies, where the spermatozoa were suspended in KRP salt solutions.

The results of the motility and per cent alive studies show that in flasks containing glycerol without fructose, motility and per cent alive were lower than in the corresponding glycerol-fructose flasks. The only exception was seen in the per cent alive studies with 10% glycerol. The most desirable motility rating was obtained with the samples containing 1% glycerol plus 1% fructose. Since more gas was produced in the 10% glycerol-fructose flasks, motility may have been depressed, due to a build-up of metabolites toxic to the cells instead of a direct toxic effect produced by the high concentration of glycerol. However, all flasks containing glycerol alone showed a lower motility, indicating a depression which probably was not associated with a build-up of toxic substances, since less metabolic activity, as measured by gas production, was observed in

these flasks than in the controls which exhibited a higher motility. This difference in motility between spermatozoa suspended in glycerol and glycerol-fructose might, in part, be explained by the report of Kampschmidt *et al.* (9), which suggests that as glucose is used to replace the buffer solution, viability of bovine spermatozoa is increased. This effect was attributed to: 1) more glycolyzable sugar present, or 2) decreased electrolyte content of the storage medium. Further support for the electrolyte view has been presented by Hendrikse *et al.* (7). They found that glycerol exerted a considerable apparent osmotic effect, with a lowering of the freezing point, when freezing bovine spermatozoa for use in artificial insemination.

One per cent glycerol in the presence of 1% fructose appeared to be more beneficial to the sperm cells, both from a motility and per cent alive standpoint, than any other combination studied. Since a rather large amount of metabolic activity was observed in these flasks without the decrease in per cent alive and motility, as observed in the 10% glycerol-fructose flasks, perhaps low concentrations of glycerol, in the presence of fructose, can be converted largely to carbon dioxide without the accompanying increase in toxic metabolic substances. Thus, a beneficial rather than a depressing effect was exerted on the motility and viability of the cells.

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TECHNICAL NOTES

ISOLATION OF IMMUNE GLOBULINS FROM MILK AND COLOSTRUM WITH RIVANOL[1]

Rivanol (2-ethoxy-6,9-diamino-acridine lactate) has been shown to precipitate quantitatively from blood serum the albumin and most of the α- and β-globulins (1, 2, 4). When applied to plasma, rivanol precipitates fibrinogen as well as albumin and α- and β-globulins. The γ-globulins remain in solution (1, 2, 4). A simple technique is thus afforded for isolation and analysis of the γ-globulins. It seemed worth while to determine whether rivanol can be employed in a similar fashion to fractionate the proteins of milk.

The technique described for blood serum or plasma was applied to separated colostrum, to acid whey from skimmilk, and to a crude lactoglobulin that had been isolated from acid whey by precipitation with 43% saturated ammonium sulfate. Specifically, the method consisted of adding 3.5 volumes of 0.4% aqueous rivanol[2] solution to one volume of solution to be fractionated, adjusting the pH to 8.5, centrifuging or filtering off the precipitated protein, and removing rivanol from the supernatant by adsorption on activated charcoal (2). The protein in the supernatant was analyzed electrophoretically in the Klett apparatus, after suitable concentration by pervaporation and dialysis against veronal buffer of pH 8.6, ionic strength = 0.1.

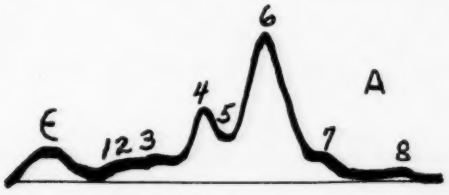
Figure 1 shows the electrophoretic patterns of crude lactoglobulin and of the filtrate obtained after treatment with rivanol. The filtrate contained primarily immune globulins, the faster components having been precipitated.

The term "immune globulins" is used herein to designate those protein fractions with mobility of -1.8 to -2.2 cm.^{2} volt^{-1} sec.$^{-1}$ at pH 8.6, ionic strength = 0.1 (4) without regard to the specific immunological properties of the components of such fractions. The components in all figures are designated numerically according to the usage of Larson and Kendall (5). Numbers 1 and 2 represent immune globulins, 4 α-lactalbumin, 6 β-lactoglobulin, 7 "blood" serum albumin, and 3, 5, and 8 the "proteose-peptone" constituents.

Figure 2 shows electrophoretic patterns of acid whey before and after treatment with rivanol. Again, the protein that was not precipitated consisted primarily of the immune globulins. The distribution of areas in Pattern B indicates that the material obtained from acid whey was 89.3% immune globulins. Furthermore, it was calculated from the electrophoretic analysis and nitrogen content of

[1] Paper No. 4071, Scientific Journal Series, Minnesota Agricultural Experiment Station. These studies were made possible in part by a grant from the Animal Disease and Parasite Research Division, Agricultural Research Service, USDA.

[2] Obtained from Winthrop Chemical Company under the name Ethodin.



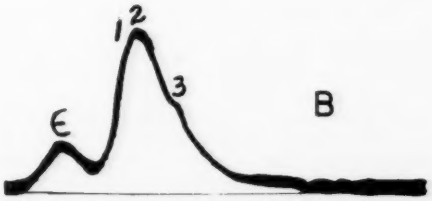


FIG. 1. Electrophoretic patterns of (A) "lactoglobulin" obtained from acid whey by 43% saturation with $(NH_4)_2SO_4$ and (B) fraction of "lactoglobulin" not precipitated by rivanol. Protein concentrations (A) = about 0.7%, (B) = 1.1%, time = 10,800 sec., temperature = 4° C., field strength = 6.00 volts/cm. Descending pattern.

FIG. 2. Electrophoretic patterns of (A) whey proteins prepared from skimmilk by removal of casein at pH 4.6 and (B) fraction of whey proteins not precipitated by rivanol. Protein concentrations (A) = 1.5%, (B) = 1.2%, time = 10,800 sec., temperature = 4° C., field strength = 6.00 volts/cm. Descending pattern.

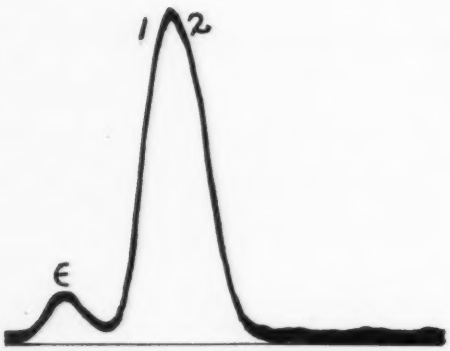


FIG. 3. Electrophoretic pattern of proteins of colostrum not precipitated by rivanol. Protein concentration = 2.8%, time = 13,540 sec., temperature = 4° C., field strength = 6.00 volts/cm. Descending pattern.

the acid whey and its rivanol filtrate that 75% of the immune globulins of the acid whey were recovered in the rivanol filtrate.

Figure 3 is the pattern for a rivanol filtrate from colostrum.

The rivanol procedure is much more simple and rapid than the classical fractionation with ammonium sulfate, as a means of isolating the immune globulins of milk. Presumably, the globulins obtained in this way are not denatured (2), although this point needs to be checked more thoroughly. Recovery of the immune globulins would need to be improved and checked before the method could be used as a basis for quantitative determination of the content of immune globulins in milk.

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INFLUENCE OF VACUUM TREATMENT UPON MILK LOSS

The advent in recent years of nonsteam-injection vacuum equipment for the removal of off-flavors from fluid dairy products has created the need for research regarding its operation. The equipment utilizes the principle of boiling off volatile components in milk by the use of vacuum. Water vapors removed in the process result in concentration or loss of milk. This note deals with milk losses resulting from processing milk in various nonsteam-injection vacuum systems at various degrees of treatment or flash-cooling.

Mixed raw milk, thoroughly agitated in a storage tank and balance tank, was pasteurized at 172° F. for 16 sec. by a high-temperature, short-time pasteurizer. The milk was homogenized at 2,500 p.s.i. after pasteurization and vacuum treatment. Vacuum treatment was accomplished using various modified vacuum installations.[1] The condenser, when used, was a 2-in.-diameter stainless steel tubular type 4 ft. long, with city water circulating spirally in the outer water jacket. The condenser was installed at a rise of approximately a 30°-angle in the vacuum-vapor line as it left the top of the vacuum chamber. In the case of the double chamber, the condenser was similarly installed, but only over the first vacuum chamber. The flow of water through the condenser was adjusted to give a 3° F. drop in temperature of the vapors passing through the condenser.

Four replicates with four different degrees of flash-cooling increments were run on each installation. Duplicate total solids determinations by the Mojonnier method were made on the treated milk and the original raw milk samples. The difference in total solids between the raw and treated samples divided by the total solids of the raw sample, multiplied by 100, resulted in the calculated percentage milk loss. The method of least squares was used for determining the linear relationship between the degree of treatment and percentage milk loss. In cases where the relationship was curvilinear, the square root of the percentage of milk loss was used to make the relationship nearly linear.

[1] Vacu-Therm made by the DeLaval Separator Company, Poughkeepsie, New York.

The standard error of estimate was used as a measure of accuracy of the line (1).

The upward trend in the percentage of milk loss, with increasing degrees of flash-cooling in the various vacuum chambers, was expressed by the following equations, where Y represents the percentage of milk loss, Y_1 the square root of the percentage of milk loss, and X the degrees of flash-cooling: (1) $Y = -0.270 + 0.109\ X$ (single vacuum chamber before final heating); (2) $Y_1 = 0.004 + 0.061\ X$ (single vacuum chamber before final heating and with a condenser); (3) $Y_1 = 0.172 + 0.047\ X$ (single vacuum chamber after the flow-diversion valve and with a condenser); (4) $Y_1 = -0.052 + 0.035\ X$ (double vacuum chamber with the first chamber before final heating and the second chamber after the flow-diversion valve); and (5) $Y_1 = -0.084 + 0.034\ X$ (double vacuum chamber as noted in four, plus a condenser). The standard error of estimate for the first equation was 0.19% milk loss, while for the remaining equations it was, respectively, 0.12, 0.15, 0.11, and 0.09 for the square root of the percentage of milk loss.

The equations for estimating the per cent milk loss from degrees of flash-cooling, in instances where a condenser is not used, are valid for similar nonsteam-injection vacuum equipment, with minor changes for radiation-loss variances. In cases where a condenser is used during treatment, the equations are not valid unless identical conditions exist, but they indicate the approximate expected milk loss.

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RECOVERY OF ADDED MONOGLYCERIDES FROM MILK

Monobutyrin is water-soluble and is probably the monoglyceride of shortest chain length that would be found in lipolyzed milk. In a previous paper (2), some of the difficulties encountered in recovering added monobutyrin from milk were described. Commercial monobutyrin contains glycerol which analyzes as if it were two moles of monoglyceride. The poor recoveries of monobutyrin encountered from milk were due to the retention of glycerol in the silica-gel when this method was used to extract monoglycerides. Thus, the difficulties centered about the purification of monobutyrin. This has been accomplished with the method of Gander (1), in which monobutyrin is eluted from a celite-silicic acid column that retains the

TABLE 1

Extraction of added monostearin and monobutyrin from milk by the silica-gel method

	Monostearin[a]		Monobutyrin[a]	
	Added	Found	Added	Found
	(mg. per 100 ml. milk)		*(mg. per 100 ml. milk)*	
2.0-m*M* level	23.00	22.30	14.94	14.10
	24.80	23.90	13.65	13.59
	23.00	22.20	13.00	12.59
	24.68	24.72	11.80	11.52
			15.77	14.98
Mean	23.87	23.28	13.83	13.36
Standard deviation of a sample[b]		0.64		0.31
Per cent recovery		97.5		96.6
0.1-m*M* level	1.27	1.27	0.75	0.69
	1.14	1.16	0.65	0.62
	1.31	1.31	0.65	0.59
	1.21	1.23	0.59	0.62
			0.79	0.86
Mean	1.23	1.24	0.69	0.66
Standard deviation of a sample		0.037		0.029
Per cent recovery		100.8		95.7

[a] Corrected for purity.
[b] Standard deviation calculated from the difference between duplicates.

glycerol. This paper describes the recovery of purified monobutyrin and monostearin from milk with the silica-gel extraction method.

Monostearin was purified by washing an ether solution with water to remove glycerol, and had a total monoglyceride content of 83.8%. Monobutyrin was purified as described above, and had a total monoglyceride content of 92.2%. Both were added to pasteurized milk at levels of 0.1 and 2.0 m*M* per 100 g. of fat. The monostearin was dispersed with a hand-operated homogenizer at 70° C. The extractions from milk and analyses of monoglycerides were performed as described in the previous paper (2). At the 2 m*M* level, 25 ml. of milk were extracted, the solvent evaporated, and the fat rinsed into a 25-ml. volumetric flask. At the 0.1 m*M* level, 50 ml. of milk were extracted and the extracted fat rinsed into a 10-ml. volumetric flask. The results are presented in Table 1. The data in Table 1 show that monoglycerides added to milk are extracted quantitatively by the silica-gel method.

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EFFECT OF SOME PRESERVATIVES ON BABCOCK TESTS AND FAT HYDROLYSIS IN SINGLE MILK SAMPLES[1]

Manus and Bendixen (4) showed that the addition of corrosive sublimate reduced the Babcock test reading and caused large increases in the free fatty acid titers of composite milk samples.

The object of this study was to compare the effect of several common preservatives on the Babcock test reading and the free fatty acid titer of milk samples stored for 1 and 2 wk.

EXPERIMENTAL PROCEDURE

In the first series of tests, seven lots of mixed Grade A raw milk from the College dairy were each split into five portions and treated as follows: (a) no additions; (b) addition of two No. 2 corrosive sublimate ($HgCl_2$) preservative tablets (0.45 g. each) per 440 ml. of milk; (c) addition of three Milkeep[2] preservative tablets (0.325 g. each) per 420 ml. of milk; (d) addition of four potassium dichromate preservative tablets per 400 ml. of milk, and (e) addition of 1 ml. Mojonnier test fluid per 455 ml. of milk.

In the second series, ten lots of mixed Grade A raw milk were each split into five portions and treated as follows: (a) 0.5 g. CP potassium dichromate per 400 ml. of milk; (b) 0.5 g. CP potassium chromate per 400 ml. of milk; (c) three tablets Milkeep per 420 ml. of milk; (d) 1 ml. Mojonnier test fluid per 455 ml. of milk, and (e) 0.6 g. CP mercuric chloride per 440 ml. of milk.

To dissolve the preservatives rapidly, all tablets were pulverized prior to addition to the milk.

Immediately after all additives were completely dissolved, milk for six Babcock test bottles was pipetted from each sample, to avoid possible errors due to fat adhering to the bottle surface after storage. Care was taken also to prevent any churning during the mixing of the sample before pipetting. Within 60 min., Babcock tests (1) were completed on the samples in two of the test bottles. They were repeated in duplicate after storage at 35–38° F. for seven and 14 days.

Triplicate samples from each preserved portion were placed in half-pint milk bottles. Immediately after the Babcock tests were completed, the free fatty acid determinations were made in duplicate, using silica gel chromatography as described by Harper *et al.* (3), except that 2 ml. of 2% phenolphthalein in absolute ethanol was used as the indicator. The free fatty acid titer tests were repeated in duplicate after storing the samples at 35–38° F. for seven and 14 days.

Differences between the average of the fat percentages obtained immediately after the additives were in solution, and the average of the percentages obtained after storage at 35–38° F. for seven and 14 days, were recorded as the observed reductions in the fat percentage during storage. The increase in free fatty acid titer was obtained in a similar manner.

The Babcock test bottles were selected for an accuracy of ± 0.05%, or less.

[1] Scientific Paper No. 1835, Washington Agricultural Experiment Stations, Pullman. Project 839.

[2] Active ingredients according to the manufacturer's label: Disodium phosphate, 3, 5-dimethyltetrahydro 1, 3, 5, – 2H–thiadiazine-2-thione.

RESULTS AND DISCUSSION

The results obtained in comparing nonpreserved and corrosive sublimate–preserved samples were essentially the same as previously reported by Manus and Bendixen (4). It did not matter whether commercial preservatives were used as in Series 1 or whether the chemically pure preservatives were used as in Series 2. Therefore, data for the second series only will be presented.

In Figure 1, the average fat percentage reduction during storage is shown to be small for the samples preserved with chromates, Milkeep, or Mojonnier test fluid, whether the holding period was 1 or 2 wk. However, mercuric chloride reduced the average Babcock percentage more than three times as much as the chromate-preserved samples, and almost doubled that for the samples preserved with Milkeep or Mojonnier test fluid.

Duncan's (2) multiple range and multiple F test shows the reduction in Babcock fat percentages to be significantly greater at the 1% level when mercuric chloride was used as a preservative than when any of the other four preservatives were used. There were no statistically significant differences in the reduction in fat percentages between the samples preserved with potassium chromate, potassium dichromate, Milkeep, or Mojonnier test fluid at the 1% level.

The increase in free fatty acids with the different preservatives as shown in Figure 1 was not statistically analyzed. However, the data show that free fatty acid increases were generally accompanied by a reduction in the Babcock fat test. The increase in free fatty acids and the reduction in the Babcock fat test were considerably greater for the samples preserved with mercuric chloride than for those preserved with the other preservatives.

Figure 2 compares both the fat percentage reduction and the free fatty acid titer increase for 1 and 2 wk. in single samples, when potassium dichromate and mercuric chloride are used as the preservatives. Generally, a larger reduction in the butterfat test is accompanied by a

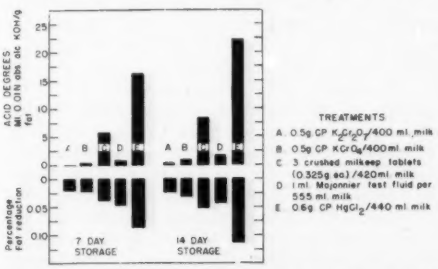
ACID DEGREES
Ml 0.01N abs. alc. KOH/g fat
Percentage fat reduction
7 DAY STORAGE
14 DAY STORAGE
TREATMENTS
A. 0.5g CP K2Cr2O7/400 ml. milk
B. 0.5g CP K2Cr2O7/400ml. milk
C. 3 crushed milkeep tablets (0.325g eq.)/420ml. milk
D. 1 ml. Mojonnier test fluid per 555 ml. milk
E. 0.6g. CP HgCl2/440 ml. milk

FIG. 1. Average increase in free fatty acids compared with average Babcock fat test reduction in ten single samples of preserved milk stored at 35 to 38° F. for seven and 14 days. (All tests in duplicate.)

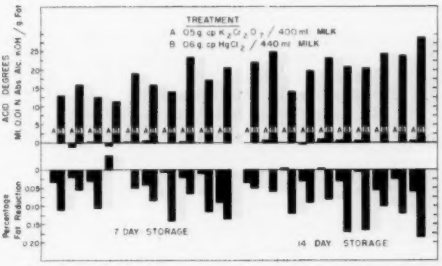
TREATMENT
A. 0.5g. cp K2Cr2O7 / 400 ml. MILK
B. 0.6g. cp HgCl2 / 440 ml. MILK
ACID DEGREES
Ml. 0.01 N Abs. Alc. KOH / g Fat
Percentage Fat Reduction
7 DAY STORAGE
14 DAY STORAGE

FIG. 2. Increase in free fatty acids compared with Babcock fat test reduction in ten single samples of preserved milk stored at 35 to 38° F. for seven and 14 days.

larger free fatty acid titer. The correlation was fair.

The reason why greater fat reductions and a large increase in free fatty acids occur with mercuric chloride is not clear.

Mercury is a protein precipitant and one may, therefore, speculate that the protein-lipid complex membrane surrounding the fat globules is precipitated by the added mercuric chloride, thus increasing the exposure of the fat to lipase activity. The mercury ion is a well known inhibitor of some enzymes. However, it appears to improve the conditions for lipolysis. Raw milk samples preserved with mercuric chloride, stored at 35–50° F. for a week or longer, invariably have a definitely rancid odor. No odor was detected in samples preserved with the chromates. The odor of samples preserved with Milkeep was that of wet phosphorus matches. Any odor that might have developed in the samples preserved with Mojonnier test fluid was masked by the formaldehyde in the test fluid.

SUMMARY

Results reported previously showing an acceleration in fat hydrolysis and a significant decrease in the fat test of milk samples preserved with mercuric chloride have been corroborated.

Milk preservatives, such as potassium dichromate, potassium chromate, Milkeep, and Mojonnier test fluid yielded results which are not significantly different at the 1% level. Mercuric chloride caused a significant lowering at the 1% level of the fat test and markedly increased the free fatty acid titer in single milk samples stored at 35–38° F. for 1 and 2 wk.

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EFFECT OF FEEDING COD LIVER OIL AND UNSATURATED FATTY ACIDS ON RUMEN VOLATILE FATTY ACIDS AND MILK FAT CONTENT

The depressing effect on the fat content of milk induced by the feeding of cod liver oil to cows has been known since the early 1920's [see review by Hilditch (2)]. Hilditch has proposed that the unsaturated fatty acids of the C_{20} and C_{22} series are responsible for this depression, possibly by exerting a poisoning effect on mammary enzyme systems. Hydrogenation of the cod liver oil eliminates the effect.

In view of high correlations observed between the molar per cent of rumen acetic acid and fat content of milk, and an equally high negative correlation between the molar per cent of rumen propionic acid and fat content of milk (9), it appeared that cod liver oil might exert its effect on fat content of milk by altering the proportions of the rumen volatile fatty acids (VFA). Accordingly, the rumen VFA of six cows were determined before and after the initiation of the per os administration of 150 ml. each morning and evening of cod liver oil, oleic acid, or linoleic acid.

All cows had been on constant feeding regimens for over a month prior to the administration of these additives. Three of the cows had been on a normal diet and three had been on diets designed to decrease the fat content of milk (1, 7). An additional cow receiving a normal diet of alfalfa hay and a mixed concentrate was fed 150 ml. of oleic acid twice per day, to ascertain the effect on the composition of milk fat. The following techniques were used: rumen VFA, a modification of the method of Wiseman and Irvin (10); iodine number, Rosenmund and Kuhnhenn (6); lower fatty acids of milk fat, a modification by Lakshmanan and Mougey in this laboratory of the Keeney method for butyric acid (3). Rumen samples were obtained 3 hr. after feeding by means of a large rumen tube (Colorado tube); the supernatant fluid obtained following centrifugation of the rumen contents was frozen for purposes of preservation and analyzed within three days.

The data presented in Table 1 show that cod liver oil, oleic acid, and linoleic acid, when administered to cows on normal diets, decreased fat content of milk and exerted a remarkable effect in (1) decreasing the molar proportion of rumen acetic acid and increasing the molar proportion of propionic acid, and (2) increasing the concentration of total VFA in the rumen fluid, linoleic acid having the greatest effect.

When given to cows on diets which had already decreased milk fat content markedly, linoleic acid once again had the greatest effect (Cow 175) on the rumen VFA, being complementary to the basal diet in decreasing the acetate and increasing the propionate proportions and in increasing the concentration of total rumen VFA. Cod liver oil also had a complementary effect (Cow 242), but to a lesser degree in effecting these changes. Oleic acid was complementary to the basal diet (Cow 164) in increasing the concentration of total VFA in the rumen fluid, but did not effect a further change in the molar proportions of rumen acetate and propionate. However, it will be noted in this case that the basal diet had already effected very large changes, the molar proportion of propionate being greater than that of acetate. The effect of feeding oleic acid on the short-chain fatty acids of milk fat is shown (Table 2). There was a decrease in the molar percentage of each of the lower fatty acids, an increase in iodine number, and a decrease in fat content of milk (25%) similar to that produced by feeding cod liver oil (2).

The effect of the additives on the fat content of milk of cows already on low-milk fat producing diets suggests some interesting possibilities. The failure to get a further depression in fat content of milk from the feeding of oleic acid (Cow 164) or linoleic acid (Cow 175) probably was due to the low levels attained on the previous diets. In Cow 242, which was fed cod liver oil, there was a slight increase followed by a decrease in the fat content of milk. The tendency of the milk fat content either to increase or undergo only slight decreases in Cows 242 and 164, in which the previous diets had effected decreases in the fat content of milk, could be explained on the basis of the earlier postulation of Shaw and Lakshmanan (8), in which it was proposed that a decreased synthesis of short-chain fatty acids (mammary lipogenesis) was ordinarily compensated to some extent by an increased uptake of longer-chain fatty acids from the blood, and vice versa. In the present instance, the fatty acids other than the C_{20} and C_{22} acids of cod liver oil may

TABLE 1

Effect of feeding cod liver oil (CLO), oleic acid (OA), and linoleic acid (LA) upon rumen volatile fatty acids (VFA) and fat content of milk

Cow	Basal diet	Additive	Time after feeding additive	Milk fat	Molar % of total rumen VFA					Total rumen fluid VFA
					C_2	C_3	C_4	C_5	C_6	
	(lb/day)	*(ml/day)*	*(hr.)*	*(%)*						*(mg/100 ml.)*
	Alfalfa hay (AH) + mixed concentrates (MC) + additives									
SK-1	22 AH + 11 MC	300 Cod liver oil	0	2.9	71.1	14.1	12.5	1.9	0.4	635.9
			63	2.4	56.1	28.2	9.1	5.2	1.4	787.8
S-1 1	12 AH + 10 MC	300 Oleic Acid	0	5.0	67.4	15.4	11.8	5.3		448.0
			63	4.5	61.4	21.5	14.3	2.7		870.1
K-1	12 AH + 12 MC	300 Linoleic Acid	0	4.0	78.9	9.9	7.7	3.4		584.2
			63	2.6	52.2	29.9	14.0	3.8		762.4
	Ground and pelleted alfalfa hay (GPAH) + heated corn (HC) or ground unheated corn (GC) + additives [a]									
242	30 GPAH + 4 GC	300 Cod liver oil	0	2.7	65.9	22.9	8.5	2.0	0.8	1,357.6
			24	2.9	62.7	26.1	7.8	2.4	0.9	1,426.2
			48	2.7	60.1	28.3	8.5	2.5	0.5	1,540.1
			72	2.5	59.6	28.4	8.5	2.8	0.8	1,549.1
164	6 GPAH + 16 HC (steam 275° F.)	300 Oleic Acid	0	1.7	43.7	47.3	3.7	4.2	1.1	948.7
			63	2.3	48.2	45.5	4.3	1.8	0.2	1,162.5
175	6 GPAH + 16 HC (Dry heat 400° F.)	300 Linoleic Acid	0	1.4	54.9	32.0	7.9	4.4	0.9	677.3
			63	1.5	46.1	41.7	7.7	3.8	0.6	1,579.1

[a] Extent of depression of fat content of milk by basal diets prior to feeding of additive: Cow 242, 25%; Cow 164, 47%; Cow 175, 59%.

TABLE 2

Effect of feeding oleic acid to a cow on milk fat composition

	Iodine number	Fatty acids of milk fat in molar %				
		C_4	C_6	C_8	$C_{10} + C_{12}$	Higher
Prior to feeding oleic acid	31.4	8.9	5.2	2.9	6.6	76.4
After feeding oleic acid three days (300 ml. per day)	49.5	4.8	2.6	2.1	4.3	86.2

have compensated to some extent for the decreased mammary lipogenesis. Oleic acid could have both an increasing and a decreasing effect, the former prevailing when mammary lipogenesis was already inhibited markedly, as in the case of Cow 164. Since linoleic acid is not normally taken up by the mammary gland in appreciable amounts, it would not be expected to have a compensatory (increasing) effect on fat content of milk.

We have noted (unpublished) that the basal milk fat depressing diets used here decrease the lower fatty acids of milk fat. These considerations would explain the variable results on fat content of milk obtained from the feeding of oils other than cod liver oil [see Petersen (5), and Moore *et al.* (4) for review]. The observations of Moore *et al.*, that fat content of milk was not decreased when cod liver oil was administered per os in small amounts but at frequent intervals, can be understood on the basis that the concentration of the C_{20} and C_{22} unsaturated fatty acids in the rumen was insufficient to produce the necessary changes in the rumen VFA.

It is conceived that the above findings not only explain the long-known effect of cod liver oil on the fat content of milk, but also offer considerable potential for influencing animal performance by controlling the proportional (and total) production of rumen VFA by means additional to those previously demonstrated (1, 7). Oils containing long-chain, highly unsaturated acids, unsaturated fatty acids per se and, possibly, certain unsaturated hydrocarbons when added to rations for ruminants, would be expected to produce the above-noted results in greater or lesser degree, depending upon the amount and degree of unsaturation of the additive. Theoretically, this should apply to pasture as well as to dry-lot feeding.

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OUR INDUSTRY TODAY

PRODUCTION OF QUALITY PROCESS STEAM

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Explanation of terms used, and review. In this presentation, the terms direct heating and indirect heating will be used rather frequently. The former refers to the direct mixing of steam with the product processed. The latter, of course, refers to steam used for heating a product through a heat-transferring surface.

If steam were used indirectly for processing dairy and food products, the only possible contamination of such foods by steam contaminants, aside from leaks, could be by residues adhering to product surfaces as a result of cleaning solutions prepared by mixing steam and water, or from sterilization of equipment by use of direct heating with steam.

Currently, steam is used directly in the dairy industry for foam heating in batch pasteurization, as a requirement by most city milk ordinances. It is also used in some vacuum-type pasteurizers for milk and cream, more recently in vacuum treatment of milk and cream to remove foreign volatile flavor and odor compounds, in the preparation of processed cheese or cheese foods, and in the preparation of some concentrated milk products, such as superheated skimmilk etc. Earlier in the dairy industry, direct steam heating was traditionally practiced for preheating milk in hot wells before concentrating.

With the advent of improved controlling equipment, and in line with newer knowledge of milk constituents, there is a tendency on the part of certain branches of the industry to favor higher processing temperatures, with correspondingly shorter holding periods.

For some time, research has been in progress to establish a time and temperature relationship when heating is carried to much higher levels than those currently suggested in the United States Public Health Service Standard Milk Ordinance and Code.

One of the principal reasons for the somewhat slow progress in this direction has been the absence of sufficiently sensitive controlling equipment. Progress is being made in this direction.

The term ultra-high heat processing has been applied to temperature treatments which are considerably in excess of the present standard for High-Temperature–Short-Time (HTST) pasteurization. Temperatures currently under consideration approach or even exceed 300° F. The time of exposure to temperatures of this magnitude would be in the nature of a fraction of a second. Even with a heavy infestation of spore formers, sterile milk has been produced at 297° F. with a 0.5-sec. holding time.

Certain problems with respect to heating equipment may be anticipated. Nevertheless, several manufacturers have equipment of this kind on the market. It is recognized that the problem could be greatly simplified if direct heating by the introduction of steam to the product were permissible. Sanitarians will have to be convinced that this type of heating is safe from the standpoint of the ultimate consumer.

Currently in this country, ultra-high heating is accomplished by use of indirect high-velocity tubular type or by swept-surface heat exchangers. Each of these minimize "burn-on," a problem with all indirect high-temperature milk heaters. Many feel that direct heating is the best answer.

The food-processing industry is using direct steam heating for various treatments. It is used for blanching and for peeling certain vegetables, for humidity control in bakery fermentation chambers, in the preparation of some fruit products such as peeling peaches, mixing with apple sauce, in the brewing industry for heating mash, etc.

Direct mixing of steam and water for cleaning and rinsing solutions is regaining some of its former popularity as a result of the development of greatly improved mixing chambers.

There is one big difference between processing dairy products and most other foods. Because of the unusual position of dairy products in the diet and their use in liquid form by infants, children, expectant mothers, and by invalids, where it sometimes constitutes the entire diet, a special problem of protection from contamination is created. Cheese or cheese foods, on the other hand, apparently are frequently classified along with fruits and vegetables with respect to tolerances for food additives.

In a number of industries where large quantities of steam are used, there has been a trend toward high-pressure generators. This necessitates specialized water-conditioning methods. New chemicals for inhibiting corrosion have been developed and, because of their effectiveness, they are also often included in water-conditioning compounds to be used in boilers operating under 200-p.s.i.g. pressure.

Water and steam conditioners as food additives. Regulatory agencies, processors, and a number of consumer groups agree that if residues from boiler water conditioners enter dairy and food products by way of direct heating, they should be regarded as additives. The Federal Food and Drug Administration undoubtedly will apply Sec. 402 (a) (1) of the Federal Food, Drug, and Cosmetic Act, which

reads in part: "A food shall be deemed to be adulterated . . . if it bears or contains any poisonous or deleterious substance which may render it injurious to health . . .;" and/or it may apply Sec. 406 (a) which reads in part ". . . except where such substance is required in the production thereof or cannot be avoided by good manufacturing practice, shall be deemed to be unsafe . . . but when such substance is so required or cannot be so avoided, the Secretary shall promulgate regulations limiting the quantity therein. . . ." Presumably, this gives the Federal Food and Drug Administration considerable latitude of interpretation. We do know that in the case of DDT, tolerances are zero for dairy products, but tolerances for fruits and vegetables may range from 1 to 5 p.p.m., depending upon the quantities of such food products present in usual diets.

The new food additive amendment to the Federal Food and Drug Act places the burden of proof of nontoxicity of a food additive on the manufacturer. I do not want to discuss the merits of this legislation but, if you are planning to use steam for direct processing, it is well to know what ingredients are present in feed-water conditioning compounds before proceeding with their use. As a result of the new amendment, we may expect new water-conditioning materials to reach consumer channels at a faster rate than was the case formerly. The manufacturer now has the alternatives of testing the product in his own laboratories or having it tested by a reputable private testing laboratory, or through the medium of an industrial grant to a recognized university or college, which will undertake to carry out the necessary tests and publish the findings.

Another factor which processors of foods should not disregard is the greatly improved techniques which have been developed or are in the process of development for detecting even minute quantities of specific additives which may be present in a food product.

Advantages and disadvantages of direct heating. A processor of dairy or food products would not be inclined to use direct steam heating unless the product can not be handled otherwise, or where it has distinct advantages. In the case of dairy products, the advantages of direct steam heating may be summarized somewhat as follows:

1) Lower processing costs, because
   (a) Smaller and less costly processing equipment may be used. On a theoretical basis, the area of a processing unit of a direct heating machine would have to be only one-eightieth of that of an indirect heater of comparable capacity.
   (b) Direct heating results in increased efficiency. This is especially true of products with a high Reynolds number (high viscosity) and turbulent flow.
   (c) Labor costs are less because cleaning operations are simplified.
2) Danger from biological recontamination is reduced.
3) Off-flavors due to high temperatures on heat-transferring areas of indirect heating equipment are reduced when direct heating is practiced.
   (a) Care should be exercised so as to avoid the use of superheated steam in direct heating, since this may result in protein denaturation and subsequent flavor defects.
4) Lower boiler pressures are possible.
5) Lower radiation losses; hence, better operating efficiencies result.

Objections to direct heating with steam include:

1) Danger of carry-over of conditioning compounds due to faulty boiler operation, such as
   (a) Overcrowding a boiler.
   (b) Foaming and priming.
2) Possibility of presence of toxic volatile corrosion inhibitors in the steam.
3) Increased amounts of make-up feed water will result in higher conditioning costs.
4) Increased scale formation will occur unless special care is exercised in conditioning the feed water.
5) More frequent blow-down or deconcentration will be necessary, thereby decreasing the efficiency of steam generation.
6) Flavor defects resulting from the presence of conditioning compounds may occur.
7) Phosphate positive reactions are more apt to result where direct heating is practiced even at the higher tempraturs, e.g., 200° F. for 0.7 sec.

An examination of the advantages and disadvantages indicate that those concerning some aspects of efficiency might cancel out. This is true of greater heating efficiency versus increased water-conditioning costs. For the time being, at least, these items will not be considered.

The immediate problem is to convince regulatory agencies and consumer groups that direct heating with steam is at least as satisfactory as, or superior to, indirect heating.

Equipment which will pass present sanitary standards for processing dairy products by direct steam heating is presently available. In the past, the problem of adequate controlling equipment for ultra-high heating limited its development. This is being rapidly overcome. Nevertheless, we must learn more about lag phases in the temperature of the various constituents in the product processed.

Steam need not necessarily contain toxic compounds in order to be objectionable. It may cause flavor or color defects, which do not con-

cern regulatory agencies. On the other hand, if it causes dilution, when such dilutions are not permissible, it may involve legal interpretations. If the product is diluted, what assurance has management that prescribed practices are followed during processing to counteract the dilution?

The plant engineer is most concerned with scaling and corrosion in the operation of a steam-generating plant. Boiler water conditioning practices are developed to overcome these problems. Most specialized treatments are generally directed toward the elimination of corrosion. High-pressure steam will usually cause more rapid corrosion than low-pressure steam. High-pressure boilers are most often encountered in steam electric generating plants, chemical plants, and industrial enterprises, rather than in dairy or food plants. For this reason, water-conditioning engineers have given much greater attention to these enterprises than to dairy and food plants.

Dissolved oxygen and/or carbon dioxide, plus improper pH control, are responsible for most corrosion problems. Some of the newer chemicals used as components of boiler feed water conditioners were developed for operations where contamination of foods was not a problem. However, when an engineer in a dairy or food plant encounters corrosion difficulties, he consults boiler conditioner manufacturers, who may not appreciate the problems of the food plant or who are not aware that steam is to be used for direct heating.

Boiler water and steam conditioners. Various techniques have been developed for overcoming the problems of scale formation on boiler surfaces or of corrosion within the boiler or in condensate return lines and processing equipment.

Conditioning of water prior to its entry to the boiler includes settling tanks, sand filters with or without coagulants, heat treatment, the use of calcium hydrate, sodium carbonate, various aluminates, and/or by the use of ion exchange equipment of various types. Electronic devices also have been advocated. For the elimination of dissolved oxygen, beds of steel shavings or sulfite treatments have been used. Reboilers also have been suggested.

For conditioning water inside of boilers a wide variety of materials is available from manufacturers of boiler compounds. The chief functions of such compounds are the replacement of objectionable water impurities by the formation of less objectionable compounds, by precipitation, or by formation of colloidal suspensions, and in some cases by film formation on susceptible surfaces as well as neutralization of oxygen and carbon dioxide activity.

Boiler water conditioning compounds (internal treatment) include sodium hydroxide, sodium carbonate, sodium sulfite, sodium polyphosphate, sodium orthophosphate, heavy phosphates, inhibited sulfanic acid, sodium alginates, quebracho,[1] phenolic type compounds such as tannins and lignins, starch, agar, gelatine, glycerine, petroleum derivatives, hydrazines, chromates, volatile or filming amines, and even sugar, whole potatoes, and cheese whey.

Contamination of steam by impurities in water or by conditioning compounds can occur by volatilization or by simple carry-over (entrainment). Volatile compounds in the form of neutralizing agents may be deliberately added to the boiler water, to protect condensate lines from corrosion. They serve a useful purpose where heating is by indirect means and the condensate is brought back to the boiler. If heating is by direct steam and no condensate is brought back to the boiler, there is less justification for the use of corrosion-inhibiting compounds. Tannins and lignins were regarded with suspicion by regulatory agencies for some time, but recently their attention has been focused on a group of compounds which includes such nonwettable film-forming long-chain amines as octadecylamine and octadecylamine acetate, and on such neutralizing amines as cyclohexylamine and morpholine. For this group of compounds there is as yet no established tolerance in dairy products consumed in fluid form. That means zero tolerance. Federal Food and Drug Administration interpretations for tolerances for additives to steam used for direct heating of food products (specifically, milk consumed in fluid form) mainly are based on naval specifications.

There is some indication that some agencies are relaxing their requirements. The Bureau of Medicine and Surgery of the U.S. Navy indicated in a letter dated July 3, 1958, that on the basis of a report from the National Academy of Science, National Research Council, the addition of octadecylamine as an anticorrosion agent in steam for cooking foods is acceptable at levels no higher than 2 to 3 p.p.m., on the basis of need or emergency. No mention is made of milk consumed in fluid form.

A more recent communication (October 13, 1958), from the Commander of the U. S. Navy Power and Utilities Branch, Maintenance Division, contained this significant statement: "On the basis of recent test data, however, it appears that this bureau may soon be in a position to relax the present restriction concerning 'food contact' on the premise that injection of octadecylamine into the system (steam) can be so controlled that its concentration in the steam will not rise to levels higher than 2 to 3 parts per million."

If the present trend toward increased application of direct heating of dairy and food products continues, it is up to the industry to develop equipment and practices which will be acceptable to regulatory agencies and to the consuming public.

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[1] South American tree, *Quebrachia lorentzii*, the bark of which has much tannin.

Handling steam without the use of toxic conditioners. A number of steps may be suggested. One is a suggestion that boilers, piping, and fittings be constructed of stainless steel. A poll of boiler manufacturers brought an almost unanimous negative reaction. First, the cost would be from eight to ten times (more for smaller boilers) higher than for carbon steel boilers. Furthermore, there appeared to be considerable doubt that the all-stainless boiler would outlast or outperform carbon steel boilers. It appears that cases of chloride stress corrosion and failure of stainless steel boilers have occurred after relatively short periods of operation. To entirely eliminate chlorides from boiler water is prohibitive in cost. Metals other than carbon steel which have been used successfully in boilers include both copper-nickel and nickel-copper alloys. These alloys have the added advantage of high-heat transference coefficients.

Corrosion of the boiler surfaces where properly conditioned water is used is slight and, hence, contamination of the steam by metals or metallic salts formed from the boiler plate is slight. There is, however, greater danger of metallic contamination from steam piping, especially if operation is on an intermittent basis. There might be some justification for the installation of stainless steel piping and fittings if direct steam heating is practiced. This would reduce the necessity for the use of corrosion-inhibiting compounds.

As a rule, the higher the pressure at which a boiler is operated, the more intense the activity of corrosion-promoting agencies. The dairy and food industry is not concerned with extremely high boiler pressures, such as 1,200 to 3,000 p.s.i.g. A pressure of 52 p.s.i.g. is sufficiently high to attain a temperature of 300°F. when direct heating is practiced, but a pressure of 100 p.s.i.g. or higher will be necessary for similar temperatures when heating is by indirect means. Neither of these pressures is considered high.

From the standpoint of solids content, steam at its worst (90% quality) would not carry in excess of 0.04% solids, but specialized steam separators or purifiers are available for installation within the boiler or steam drum, or between the boiler and processing equipment, to remove even such solids. Traps and strainers in the line are standard in most installations. These accessories can be designed to reduce the solids carry-over in steam to approximately 1.0–2.0 p.p.m., when the boiler is operated at less than 3,500 p.p.m. of solids in the water. With highly specialized separators the solids carry-over may be brought down to 0.3 p.p.m.

The safety of additives in boiler water or to steam depends upon management and the plant engineer. Boiler feed-water conditioners or steam additives should be purchased only after checking with management and the plant laboratory.

Management should consult with the plant engineer to assure a boiler of ample capacity, so that it need not be forced. The boiler should have the proper design for a possible fluctuating load. Boiler salesmen should not recommend a type or size of boiler which obviously is not suited to the purpose for which it is to be used.

In the installation of steam lines, lead or other toxic jointing compounds should be avoided. The boiler and the steam lines should be thoroughly cleaned before the plant is placed in operation for direct heating.

The blow-down of the boiler should be carefully watched, so that over-concentration and foaming are avoided. In many cases, a continuous blow-down system has an economical advantage. For dairy and food plants a solids concentration of 3,500 p.p.m. in the boiler should be considered as maximum.

Some dairy and food plant architects, for reasons of their own, have advocated desegregation of steam-generating equipment by placing automatic-type boilers adjacent to various processing areas. The same idea could be extended to the use of a special boiler for producing steam to be used for direct injection. This would permit a water-conditioning procedure to exclude toxic compounds of any kind.

Where steam for all purposes is produced in one common steam-generating plant, and if corrosion of condensate return lines is a problem, certain precautions may be observed. The substitution of starch for other corrosion inhibitors, such as tannins, lignins, chromates, and amines, may serve the purpose with somewhat less desirable results. Special phosphate-alkaline formulations have been developed for direct injection into steam lines for film production and acid neutralization. In the amounts necessary, they are nontoxic. Special alloys for condensate return lines might be advisable.

Since oxygen and carbon dioxide are mainly responsible for corrosion, special techniques might be used for removing these gases before the feed water enters the boiler. Some of these have been previously indicated. One concern which required steam of exceptional purity solved the problem of corrosion and scaling by installing a two-stage steam generator. A gas-fired heater is used to heat a refined heat-transfer oil to 500° F. The hot oil is passed through a tubular heat-transfer steam generator for converting 200° F. preheated water into steam. From the oil-heated steam generator, the oil which still carries heat passes through a double-jacketed water heater in which the cold feed water is preheated to 200° F. Scale will form on the surface of both heaters, so that construction must be such that they can be easily opened for descaling.

Sodium sulfite is frequently added to boiler feed water as an oxygen-reducing agent. The amount may range from 20 to 50 p.p.m. The concentration in the carry-over could reach 200 to 500 p.p.m. Sulfites are permitted as ad-

ditives in a number of foods and, hence, should not be regarded as too objectionable for use in boiler water treatments, except in the case of milk consumed in fluid form.

One aspect of the oxygen problem is that of prevention. Improperly sealed centrifugal condensate return pumps may add from 200 to 600 p.p.m. of oxygen by volume. Water itself may be a contributing factor. Oxygen solubility may reach 16,000 p.p.m. by volume, but probably averages closer to 6,000 p.p.m. For most boiler-operating conditions, it should be kept below 30 p.p.m. by volume in the boiler, although some operators insist that it may reach 100 p.p.m. without causing a serious problem.

Proper adjustment of the pH of boiler water is helpful in reducing corrosion. Iron is least soluble at pH 9.6, but this is unsatisfactory for other metals or alloys in a steam system and, hence, it is customary to maintain a pH of 8.6–9.0.

The discussion up to this stage has been based on the assumption that any toxic corrosion inhibitor must be kept completely away from the product, which is heated directly with steam. If steam were 100% in quality, none but volatile compounds would be carried over. Suppose, however, that it were 95% quality and that the solids concentration of the 5% carry-over were 3,500 p.p.m. If such steam were at 52 p.s.i.g. pressure, and if it were used to heat milk directly from 35 to 300° F. with no attempt to use a steam separator or traps, the amount of solids added to the milk-condensate mixture would amount to 32 p.p.m. If an amount of water equivalent to the condensate were then evaporated from the mixture, the solids added by the steam would be slightly under 40 p.p.m. in the product.

Toxicity of amine types of conditioners. Where filming amines such as octadecylamine are added, the usual dosage is at the rate of 1 to 3 p.p.m. of steam and, hence, the concentration in the milk condensate mixture would be less than 1 p.p.m.

Extensive studies have been done on the toxicity of octaldecylamine by Dr. W. B. Deichmann,[2] who is quoted as follows: "No toxic effects whatever, due to octadecylamine, were observed when this compound was fed in the diet to rats for two years at levels up to 500 p.p.m. The weight gains, blood counts, survival, and food consumption of the rats fed octadecylamine were indistinguishable from the controls. No significant pathological changes due to octadecylamine were observed when the tissues of these animals were examined microscopically."

[2] University of Miami, School of Medicine, Coral Gables, Florida.

Dogs likewise were used in the experiments, and the final statement follows: "Thus it would appear from this investigation that the maximum nontoxic levels of octadecylamine are 500 p.p.m. for the rat and 3.0 mg/kgm of body weight per day (equivalent to approximately 100 p.p.m. in the diet) for the dog."

Attitude of Federal Food and Drug Administration. The Food and Drug Administration, Department of Health, Education and Welfare, in a letter dated October 21, 1958, stated their position in the following words: " . . . As a matter of general policy, we of course take the position that where special additives are used they should not result in the adulteration of foods, drugs, or cosmetics.

"We have had occasion to consider the suitability of cyclohexylamine and morpholine as anticorrosive agents for steam lines where the steam may contact food. We have found that where the steam does not contain more than 10 parts per million of the amines representing the combined and free amount of either, there is no hazard to health."

This letter has been supplemented under date of December 9, 1958, with another, which reads in part " . . . We have not sanctioned the use of any anticorrosive agent in steam which would contact fluid milk.

"We have not sanctioned the use of any ammonia compounds for use in boiler water under the conditions you describe. We would think that such substances would be food additives as described in the Food Additives Amendment and should not be used under conditions whereby they might be introduced into food, except pursuant to an order issued by this Administration, which would, of course, have to be based on a petition filed by some interested person."

The purpose of this discussion was to point out the advantages and disadvantages of direct heating with steam and the obstacles involved. Solutions to problems which would be encountered were suggested on a generalized basis. Each branch of the dairy and food industry would have peculiar problems of their own which would have to be taken care of locally. Water treatments depend upon water conditions and, hence, generalization is all that can be undertaken in a discussion such as this.

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PROBLEMS IN COTTAGE CHEESE PRODUCTION FOR 1959 [1]

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The biggest problem facing the cottage cheese industry in 1959 is the same as it was in 1958—that is, to make the best cottage cheese in the most efficient manner, to get it to the consumer in the shortest time possible with the best flavor, and at a price that will encourage the consumer to eat more cottage cheese than ever before. Although shelf life of cottage cheese has been greatly prolonged, it does not improve with age. Freshness is still a premium factor.

Let us enumerate the important steps, not necessarily in order of importance, that will help achieve the stated objectives. What is the first requirement for high-quality cottage cheese? It is high-quality milk of good flavor and good keeping quality. Need it be Grade A milk? Not necessarily—in some markets it might need to be Grade A, but it does not follow that only Grade A milk is of fine flavor or good keeping quality. Furthermore, let us ask the following questions and give brief answers:

(a) Is Grade A milk safer than ungraded milk? It should be, if Grade A regulations are properly enforced.

(b) Will cottage cheese made from Grade A milk protect the public health better than that made from ungraded milk? It is possible, although properly pasteurized milk is safe unless heat-stable toxins have been produced in contaminated raw milk.

(c) Is Grade A milk lower in bacterial count than ungraded milk? It is probable, but not necessarily so.

(d) Does Grade A milk have a better flavor than ungraded milk? Not necessarily so.

(e) Is Grade A milk more likely to be free from mastitis infection than ungraded milk? Not necessarily so.

(f) Is Grade A milk more likely to be free from antibiotics than ungraded milk? No.

(g) Is Grade A milk more free from sediment than ungraded milk? Not necessarily so.

(h) Is Grade A milk stored at a lower temperature than ungraded milk? Probably, but not always.

(i) Is Grade A milk produced under more sanitary conditions than ungraded milk? Yes, if Grade A regulations are properly enforced.

(j) Is Grade A milk more likely to be free from rancid flavors than ungraded milk? No.

(k) Can you tell by eating cottage cheese whether it is made from Grade A milk or not? No—probably not.

[1] Presented at Dairy Technology Conference, University of Illinois, March 25 and 26, 1959.

The use of poor-quality milk is not being advocated. However, it is true that Grade A milk is not the only kind of milk that can be used for good cottage cheese, because at present a relatively small percentage of cottage cheese is being made from Grade A milk. The consumption of cottage cheese now is at an all-time high. Nevertheless, it is necessary to have milk, regardless of its label, which is of good flavor—free from feed, malty, unclean, rancid, and other off-flavors, that carry over to the cheese curd. It must be free from antibiotic or inhibitory substances; it should be of such bacterial population that after pasteurization it will have a good keeping quality; it should be clean, and it should be free of toxins and pathogenic organisms.

The second requirement is that the milk be of normal composition— free from adulteration. High solids skimmilk of 9.3 to 9.5% is preferred to low solids skimmilk, to secure maximum yields. It is possible to recover 38–39% of T.S. of the skim in cottage cheese with high solids milk, but only 31–33% if low solids milk is used. Maximum profits can be secured only if maximum yields are secured. Fortification of skimmilk is generally practised today, to be assured of high solids. Pasteurization should follow restandardization of solids by nonfat dry milk solids. In other words, powder should not be added to the skimmilk at the vat at time of setting. Standardization of the milk using a lactometer is a good practice. A reading of 37 to 38° lactometer reading is satisfactory. Yield of cheese per 100 lb. skimmilk can be calculated from the formula:

$$\text{Yield} = 4.9 \times [\%\ \text{total solids in skim}] - 29.7.$$

For example: Skimmilk has 9% T.S. Then $[4.9 \times 9] - 29.7 = 14.4$. Yield of dry curd containing 80% moisture should be very close to 14.4 lb./100 lb. skim.

A third requirement is that the skimmilk be properly pasteurized. Although excessively high pasteurization temperatures are used for many dairy products, the minimum temperature that provides for negative phosphatase is best for cottage cheese milk. The most desirable type of body and texture is obtained from normal heat treatment. Lower yields due to shattering can be expected from milk heated above normal pasteurization temperatures, as well as securing cheese having a weak body and texture.

The pasteurized milk should have a low bacterial count if good keeping quality in the cheese is to be expected. Generally speaking, the original milk should have a low bacterial

count and small numbers of heat-resistant bacteria if the pasteurized count is to be low.

The fourth factor of importance is trained personnel and adequate manufacturing facilities. High-quality cottage cheese is a specialized, competitive product that must be made by experts who have proper facilities for doing a satisfactory job. Many dairy companies today are finding it more economical to buy their cottage cheese from companies which specialize in cottage cheese manufacture, than to make it themselves. The cost of milk, cost of labor, cost of new modern equipment, the problems of culture control, antibiotics in milk, bacteriophage, slow sets, lack of adequate refrigeration, poor keeping-quality of cheese, have all been factors in bringing about consolidation or integration of cottage cheese manufacture. Certainly, the trend is for larger and larger cottage cheese operations. Larger plants will install labor-saving and automatic equipment as soon as it is available and it will fit their operating needs.

The fifth factor of importance is culture control. If one is to make a cultured product, but can not maintain cultures, he might as well quit, for he will have to sooner or later. Volumes of literature have been published on starters, and many years have been devoted to research on starters, but as yet we do not have all the answers. Nevertheless, this should not deter us from using the best information we have available on starter care and propagation.

Important steps in maintaining cultures: (a) Secure an active starter culture; (b) have a good milk supply free from antibiotics—tested reconstituted nonfat dry milk solids provides a good culture medium; (c) use proper sterilization procedures for the milk—20 lb. pressure for 10 min., or 190° F. for 1 hr.; (d) maintain proper temperature (70–72° F.) for incubation; (e) transfer in a phage-free atmosphere; (f) incubate for 12 hr.; (g) cool to 40° F. after incubation, (h) maintain supply of frozen cultures or milk inoculated with culture and then kept frozen ready to be incubated.

Temperature control during incubation is probably the most important single factor in maintaining uniformity in cultures.

The sixth factor is know-how in the actual manufacturing procedures. If possible, use a short-time set or some modification of it to reduce growth of contaminants during incubation. Use a minimum amount of starter to give desired acidity in the allotted time. Adjust incubation temperature to 78–86° F., depending on work schedule. Use rennet extract at the rate of 0.6–0.8 ml/1,000 lb. skimmilk or coagulator at equivalent rate, so as to avoid early coagulation by enzyme without acidity. Cut curd at proper titratable acidity, depending on composition of milk and size of curd particles desired. Proper pH may be as high as 4.8 or as low as 4.65, depending on curd size, size of vat, and rate of heating. Acid development during manufacture is desirable and necessary, but excessive development is harmful. Many lots of cheese have become soft and yields have been reduced because excessive acid was developed during the initial stages of heating. An increase of 0.05–0.07% is desirable, and hot water can be used to dilute the acid concentration if necessary.

Careful and controlled agitation is essential during cooking so as to secure uniformity in heating. However, excessive agitation promotes low yields and lack of uniformity in curd size.

Cooking of the curd is extremely important and is a determining factor in the keeping quality of the cheese. Cook at 125 to 135° F. for sufficient time to firm the curd. This may take 30–60 min. Cooking temperature is controlled by pasteurization temperature and acidity. High pasteurization temperature (165° F.—16 sec.) and high acidity (pH 4.5) require high cooking temperature.

After cooking, cool the curd gradually by adding wash water. Use three waters at 80, 60–65, and 35–40° F. Whey is removed from the interior of the curd by the action of the water and the curd is cooled—both are necessary. The water used should be chlorinated or sterilized in some manner, and the third water, particularly, should be adjusted in pH to 4.8–5.0 by the addition of a food-grade acid like hydrochloric or phosphoric. Excessive aroma can be developed in the cheese if citric acid is used. Phenol and medicinal-like flavors can be very troublesome problems in areas where surface water is used. Carbonated beverage plants can not use such water and neither should cottage cheese plants. There should be approximately 20-min. intervals between addition of waters. The final water may remain on the curd 30 to 60 min., depending on how completely the curd is chilled.

Final draining may take 30 to 60 min., depending on the condition of the curd. The vat should be cooled with 32° F. sweet water in the jacket during draining and creaming.

Dressing should be applied to the curd after it has been drained, to maintain its desirable meaty body. The dressing usually constitutes 33–35% of the finished cheese. The cheese contains 0.9–1.0% salt. If the dressing is colder than the curd, it will penetrate into the curd rapidly. Usually, 1 hr. is required for dressing absorption before packaging can be done, if the cheese is packaged from the vat. Otherwise, uniformity in composition can not be maintained. If a known weight of curd is placed in a can containing a known weight of dressing, usually packaging is delayed until the following day. Temperature at packaging should be 50° F. or below.

Sanitation control, although listed as seventh, is of prime importance. Unless excellent sanitation practices are followed, the shelf life may

TABLE 1

Growth of added coliform organisms during incubation, manufacture, and storage of cottage cheese [a]

Stage of manufacture — Lot number	1	2	3	4
Time and temperature of incubation	12 hr. at 72° F.	12 hr. at 72° F.	6¼ hr. at 86° F.	5 hr. at 86° F.
		(No. per ml. or g. of cheese)		
Number organisms added/milliliter milk	500	600	3,500	1,500
Number organisms at cutting curd (pH 4.6)	< 1,000,000	46,000	23,000	(pH 4.8) 16,000
Curd after reaching 105° F.	.....	240,000	.....	900,000
Whey after reaching 105° F.	.....	2,600	.....	7,000
Curd after reaching 110° F. (pH 4.55)	140,000	114,000	24,000	130,000
Whey after reaching 110° F.	0 in 10^3 dilution	1,400	6,000	5,000
Curd after reaching 120° F.	8,000	80	100,000	(pH 4.7) 110,000
Whey after reaching 120° F.	0 in 10^3 dilution	1	29	4,000
Curd held 30 min. at 120° F.	0 (1–10)	0 (1–2 dilution)	25 (1–2)	110,000
Whey held 30 min. at 120° F.	0 (1–10)	0 (1 ml.)	0 (3 ml.)	200
Curd after reaching 130° F.	0 (1–2)	0 (1–2 dilution)	1 (1–2 dilution)	60
Whey after reaching 130° F.	0 (2 ml.)	0 (1 ml.)	0 (3 ml.)	0 (in 3 ml.)
Dressing	0 (1–2)	0 (1 ml.)	3 (10/3 ml.)	0 (in 2 ml.)
Dry curd after washing (3 hr. after cutting)	0 (1–2)	(1–2)	0 (1–2 dilution)	0 (3 ml. 1–2)
Creamed cottage cheese (pH 5.2–5.3)	0 (1–2)	0 (1–2)	1 (1–2 dilution)	0 (5 ml. 1–2)
After 2 days at 60–62° F.	.....		200,000	4 (1–2)
After 4 days at 60–62° F.	.....	60,000,000	55,000,000	
After 7 days at 60–62° F.	> 19,000,000	40,000,000 (pH 4.78)	43,000,000	1,300,000 (14 days pH 4.95)
After 4 days at 48–50° F.	.....	20,000		
After 7 days at 48–50° F.	.....	2,000,000 (pH 5.23)		
After 2 days at 38–40° F.			4 (1–2 dilution)	0 (1–2)
After 4 days at 38–40° F.		0 (1–2 dilution)	6 (1–2)	
After 7 days at 38–40° F.		8 (1–2 dilution)	4 (1–2)	
After 14 days at 38–40° F.			6 (1–2)	0 (3 ml.–1/10)
After 14 days at 38–40° F. (dry curd)		0 in (1–100 dilution)		
After 21 days at 38–40° F.	2 (1–2) good flavor (pH 5.28)		1 (2 ml.–1/10)	0 (3 ml.–1/10) (slightly bitter)

[a] Acknowledgment is given to Yasuo Azuma for making the bacteriological analyses.

be only two instead of 21 days. The following factors are important in determining shelf life of cottage cheese: (a) Original bacterial count of milk and the number surviving pasteurization; (b) contamination occurring following pasteurization—pipes, vats, agitators, etc.; (c) growth occurring during period of incubation; (d) number or proportion of bacteria killed during cooking of the curd; (e) contamination following cooking, which is added by equipment and wash water; (f) number and kinds of organisms added by the dressing; (g) number and kinds of organisms added by storage cans, agitators, shovels, scoops, pipes, pumps, and packaging machine, (h) temperature of cheese at packaging and temperature maintained during storage.

In some areas, there are public health ordinances requiring that cottage cheese have less than ten coliform organisms and less than ten yeasts and molds per gram of cheese. To study the growth characteristics, as well as their resistance or lack of resistance to the cheese-making process, four lots of cheese were made, to which coliform organisms were added to the pasteurized milk. These organisms were then followed throughout the process of manufacture and storage of the cheese. The results of this study are given in Table 1 and reveal the following:

(a) In spite of heavy inoculation, growth during incubation was not great enough to cause an off-flavor or a gassy, pin-hole cheese to be produced.

(b) These organisms are almost completely eliminated by the cooking at 130° F. It is possible to get zero count/gram cheese in a (1-2 dilution). Organisms are killed more quickly in whey than in curd.

(c) Rate of growth of those coliforms surviving cooking is rapid at 60–62° F., is less rapid at 48–50° F., and at 40° F. is almost negligible. In Lot 1, after 21 days at 38–40° F., the coliform count was 2 (1-2 dilution), but at 60° F. it was more than 10,000,000 in seven days.

(d) Growth of yeast and molds and psychrophiles was not a problem at 40° F., but would cause concern at 48–50° F. or 60° F.

(e) It is possible to secure a zero count in a (1-2) or 1-10 dilution on fresh cheese under the following conditions: 1. Care is exercised during the creaming and packaging of the cheese; 2. cheese is stored at 40° F. or below, 3. sample is secured shortly after packaging.

What is the significance of coliform organisms in cottage cheese? Coliform organisms in cottage cheese have no public health significance other than indicating the sanitary condition of the equipment, the sanitary technique employed during manufacture, and perhaps the sanitary knowledge of the cheese-maker.

Sterilization of all equipment used in conjunction with cottage cheese manufacture is essential. Utensils used in the manufacture of cheese should be taken directly from a sterilizing tank at the time of use and again immersed in the sterilizing medium when not in use. This procedure, of course, applies during the manufacturing operation.

The cheese-maker should be a careful operator. He should wear clean clothes, have a chlorine solution available for washing his hands when he handles paddles, scoops, etc. Single-service towels should be provided.

TABLE 2

Yeasts, molds, and psychrophiles per gram cheese

Conditions	Yeasts	Molds	Psychrophiles
		Lot 1	
Fresh cheese	0 (1–2)	0 (1–2)	0 (1–2)
After 7 days at 60° F.	12,000	50	Less than 10
		Lot 2	
Fresh cheese	0	0	0
After 7 days at 60° F.	15,000	0	8,600,000
After 5 days at 48–50° F.	3 (1–2)	3 (1–2)	
After 7 days at 48–50° F.			6,300,000
After 5 days at 38–40° F.	0 (1–2)		Less than 10
		Lot 3	
Fresh cheese	0 (1–2)	0 (1–2)	Less than 10
After 7 days at 38–40° F.	.....	.....	.....
After 14 days at 38–40° F.	0 (1–2)	0 (1–2)	5,000
After 21 days at 38–40° F.	1,000	66 (1–2)	Less than 5,000
		Lot 4	
Fresh cheese	0 (1–2)	0 (1–2)	Less than 10
After 7 days at 38–40° F.	.....	.....	.....
After 14 days at 38–40° F.	0 (3 ml.–1/10)	0 (3 ml.–1/10)	Less than 10,000
After 21 days at 38–40° F.	0	20 (2 ml.–1/10)	Less than 10,000

The outside of the vat should be kept clean, otherwise contamination can occur in the following cycle—outside of cheese vat→clothes→hands→utensils→cheese.

The following is a list of important sources of contamination by organisms that cause spoilage in cottage cheese:

Psychrophilic Organisms

1. Wash water
2. Cheese vat
3. Agitators, knives, paddles, etc.
4. Dressing or creaming mixture
5. Cans used to store cream, dressing, or curd
6. Homogenizer
7. Pipes
8. Filler or packaging equipment
9. Curd scoops
10. Vegetables used to flavor cottage cheese
11. Other equipment or products, not sterilized, used in cottage cheese manufacture
12. Direct contamination by cheese-maker

Coliform Organisms

1. Pipes
2. Cheese vat
3. Agitators, knives, paddles, etc.
4. Dressing or creaming mixture
5. Cans used to store cream, dressing, or curd
6. Homogenizer
7. Filler or packaging equipment
8. Curd scoops
9. Vegetables used to flavor cottage cheese
10. Other equipment not sterilized, or products not handled in approved manner
11. Direct contamination by cheese-maker

Yeasts and Molds

1. Pipes
2. Cheese vat
3. Agitators, knives, paddles, etc.
4. Dressing or creaming mixture
5. Cans used to store cream, dressing, or curd
6. Homogenizer
7. Filler or packaging equipment
8. Curd scoops
9. Cartons and lids
10. Vegetables used to flavor cottage cheese
11. Other equipment not sterilized, or products not handled in a sanitary manner
12. Direct contamination by cheese-maker
13. Air-borne contamination

Cottage cheese is an excellent food. It is one of our cheapest and best sources of proteins. It is looked on very favorably by the medical profession at the present time. The public is eating more cottage cheese today than ever before. There have been no epidemics traced to cottage cheese made from pasteurized milk. There apparently have been no food-poisoning cases traced to cottage cheese. The dairy industry can be proud of what it has achieved in improving the quality of cottage cheese. This has been brought about by competitive forces within the industry and by the increased level of understanding of sanitation and public health by dairy plant operators. The consumer confidence and acceptance that the dairy industry has, is certainly due in part to the confidence that the public has in public health officials. Let us continue to do those things which will continue to give the consumer the best cottage cheese at a fair and reasonable cost.

BOOK REVIEWS

THE HANDBOOK OF FEEDSTUFFS. Rudolph Seiden and R. H. Pfander. Springer Publishing Company, Inc., New York, 1957.

This book is a very abbreviated encyclopedia covering the grains, the minerals, and the plant and animal by-product feeds. In addition, brief botanical descriptions of most of the plants which are fed in the United States are provided, and the pests and diseases affecting them are mentioned along with suggested control measures.

Pharmaceuticals and feed additives are listed and their use briefly described. Terms commonly used in feeding are defined very simply.

Considering the tremendously broad subject matter areas encompassed in the book's subtitle, Production, Formulation and Medication of Feedstuffs, it is easy to comprehend the somewhat inadequate explanation found in the text.

This book can serve as a reference for workers in the feedstuffs industry and possibly for others concerned in the general area of feeding.

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MEAT HYGIENE. A. R. Miller. Second Edition. Lea & Febiger, Philadelphia. 557 pages.

This book is intended as a text for students of meat hygiene or for those who are engaged in the inspection of meat and poultry animals. It is, however, sufficiently comprehensive and authoritative to be a desirable reference work for all who are concerned with the processing of meat and poultry products.

The varied material of this book is presented in 17 chapters. The first two chapters establish a background for the remainder of the book by discussing History and Elements of Meat Hygiene, the latter chapter being rather brief, for establishing a proper perspective on the importance of meat hygiene. Chapters 3 and 4 describe the techniques and the pathology involved in the ante-mortem and post-mortem inspection of both meat animals and poultry and, together with Chapter 8 on Facilities for Inspection, adequately take care of this phase of the subject matter. The problem of trichinae in pork products is presented in Chapter 5; the discussion is supplemented with the reproduction of important reports on trichinosis and summaries of several important court cases which involved infected pork meat. Chapter 6, entitled Physical and Chemical Characteristics of Meat and Principal Organs, provides a general background in the histology and chemistry of meat.

The very important problem of sanitation in plant operation is handled in Chapter 7, one of the longer chapters of the book. The first half of this chapter deals with fundamentals of sanitation, including water supply, waste disposal, insect and rodent control, detergents, and disinfectants; whereas, the remainder largely describes equipment but does include some meat technology. Additional technology is supplied in Chapters 9 and 10. Chapter 9, entitled Materials Added to Meats, of necessity must be somewhat encyclopedic in nature, but one questions the necessity of some of the historical and descriptive detail such as is used to describe the various sugar preparations. In Chapter 10, the author provides a concise account of the handling of carcass meat, meat processing, and the preparation of a variety of meat products. Meat and poultry grading is outlined in Chapter 11. Chapter 12, Adulteration and Misrepresentation, Chapter 13, Deteriorative Changes in Meat, and the final chapter (17) Organized Meat Hygiene Control supplement the earlier discussion on sanitation and emphasize the legal aspects of the inspection programs.

The three new chapters on Food Poisoning, Chemical Additives, and Ionizing Radiation are brief but helpful in providing a general background on these subjects, and are desirable towards completing this discussion on food or meat hygiene.

The book is well written. The illustrations and diagrams are clear and well chosen. It is relatively free of typographical errors or other minor mistakes, although this reviewer was somewhat chagrined to read a three-paragraph discussion on *Escherichia coli* under the printed subheading *Enterococcus*.

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BREEDING 10,000,000 CATTLE. Production Division, Milk Marketing Board, Thomas Ditton, Surrey, England.

This book is a synoptic history of the artificial insemination service operated by the Milk Marketing Board. From a rather modest beginning in 1944-45, in which year approximately 2,600 cows were bred, the service grew rapidly to 1957-58, in which year approximately 1,399,000 cows were bred. At the end of December, 1958, the Board's A.I. service had bred 10,000,000 cows; thus, the title of the book.

In addition to this rapid growth, the A.I. service of the Milk Marketing Board should be proud of its committee organization, its methods of choosing and testing bulls, its plan of rearing bull calves, and its insemination fee of one pound. The material on selection pro-

cesses and rearing of young bulls, pages 7-13, inclusive, should be of interest to all A.I. organizations.

A.I. service is available from 15 breeds, of which four are dairy, four dual purpose, and seven beef breeds. During the 14 yr., there have been major changes in the numbers bred to bulls of the various breeds. On pages 20 and 21 are listed the numbers of cows bred per year to the various breeds of bulls. Two interesting facts about this table are the early and rapid increase in the numbers bred to Friesian bulls and the later and almost as rapid increases in the numbers bred to Aberdeen Angus and Hereford bulls. It is suggested that these result from a wish to obtain higher milk yields and an attempt to breed for a cross that will meet demand either for size or quality in producing beef. In more recent years, the A.I. service has emphasized the production of beef from the dairy herd.

The history of the A.I. service of the Milk Marketing Board is a story of success, and in the book, Breeding 10,000,000 Cattle, that story is told briefly and well.

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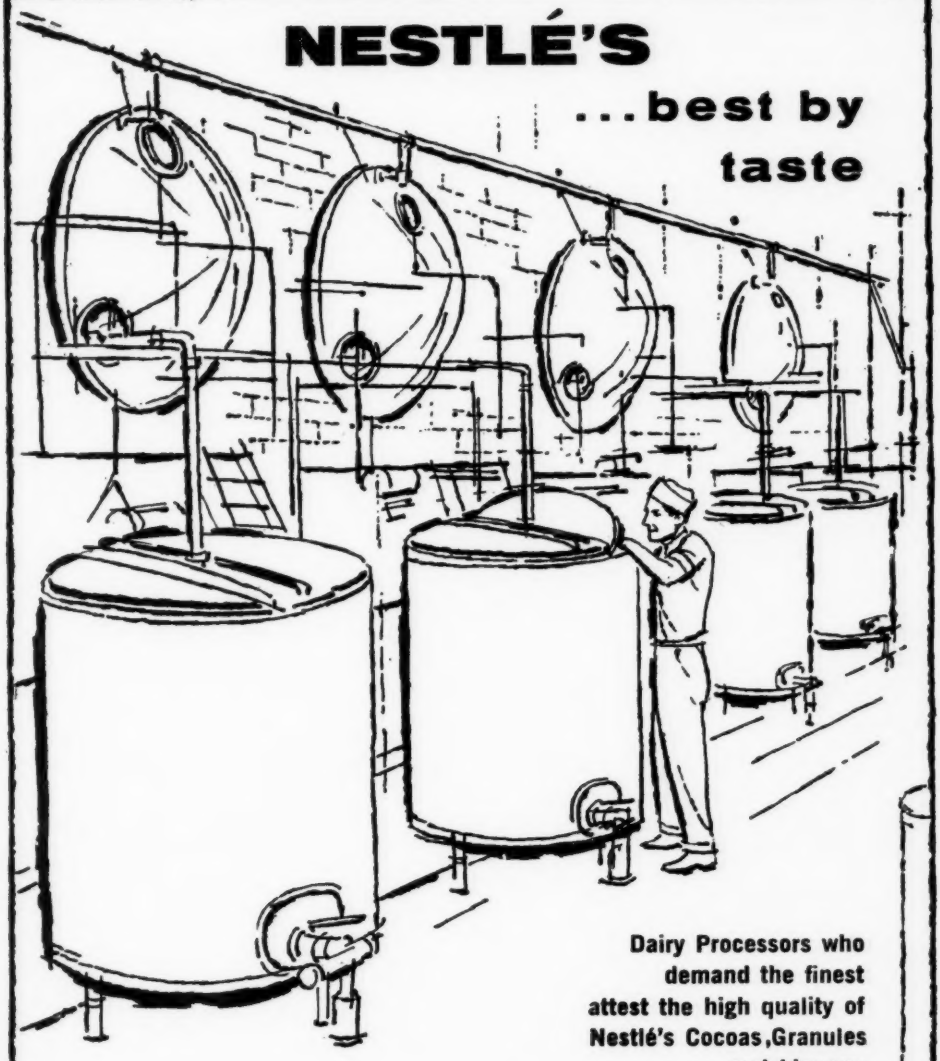

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